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(54) Title: REGULATED TARGET EXPRESSION FOR SCREENING

(57) Abstract

Methods and compositions for screening compounds for potential therapeutic activity and for identifying drug targets are provided. The methods rely on controlled expression (either underexpression or overexpression) of an essential cellular gene, which can be achieved, in one embodiment, by fusion of a heterologous regulatory element to the gene. The method is capable of identifying a drug target in the absence of any knowledge of target function.

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5 **REGULATED TARGET EXPRESSION FOR SCREENING**

CROSS-REFERENCE TO RELATED APPLICATIONS

 This application claims priority under 35 U.S.C. § 119(e) to the following
U.S. Provisional Patent applications: 60/098,563, filed April 14, 1998;
10 60/082,952, filed April 24, 1998; 60/100,430, filed July 10, 1998; 60/105,441,
filed October 23, 1998; 60/105,447, filed October 23, 1998; 60/117,758, filed
January 29, 1999 and 60/117,955, filed January 29, 1999. The disclosures of all
of these applications are hereby incorporated by reference herein in their
entireties.

15

TECHNICAL FIELD

 This invention is in the field of drug screening and drug discovery. More
particularly, techniques of microbial genetics are utilized to provide methods and
compositions for identifying targets and screening candidate therapeutics.

20

BACKGROUND

 Many methods exist for the discovery of novel therapeutic agents, such as
antibiotics. Cell-free, target-based assays often identify potent target inhibitors,
but inhibitors identified in this fashion often exhibit no activity or only poor
25 activity against whole cells. *See*, for example, Isaacson (1994) *Exp. Opin.*
Investig. Drugs 3:83-91; J. Sutcliffe and N. Georgopapadakou (*eds.*) *Emerging*
Targets in Antibacterial and Antifungal Chemistry, Chapman and Hall, London,
1992. Whole-cell methods have traditionally involved screening compounds
against wild-type strains of pathogens and selecting as candidates those
30 compounds which have a negative effect on the viability of the pathogen. Under

these conditions, compounds selected as candidates generally interact with a target that is expressed at wild-type levels. The potential of this type of assay is limited, since it provides no information on mechanism of action, which is critical for selection of a candidate. Identification of drug targets and determination of target function are costly and time-consuming processes. One approach to overcoming some of these problems has been to isolate mutants that are hypersusceptible to a particular agent and use them to screen for new agents having similar properties and/or mechanisms of action. In addition, compounds having activity against a hypersusceptible strain can often, with minimal modification, be converted to agents with strong activity against the wild-type strain. Such hypersusceptible mutants are generally obtained following standard chemical mutagenesis with agents such as N-methyl-N'-nitro-N-nitrosoguanidine. See, for example, Kitano *et al.* (1977) *The Japanese Journal of Antibiotics*, vol. XXX Suppl., pp. S239-S245; Numata *et al.* (1986) *The Journal of Antibiotics*, vol. XXXIX, pp. 994-1000; and Kamogashira *et al.* (1988) *The Journal of Antibiotics*, vol. XLI, pp. 803-806.

Systems for regulated expression of cloned genes have been described. These include the following promoters: *trp*, *lpp*, *lac*, *tac*, *trc*, λP_L , λP_R , *tetA*, *recA*, *phoA*, *malX*, *malM* (*S. pneumoniae*), *xyl* (*S. carnosus*) and T7. See, for example, Tacon *et al.* (1980) *Mol. Gen. Genet.* 177:427-438; Ghayeb *et al.* (1984) *EMBO J.* 3:2437-2442; Germino *et al.* (1983) *Cell* 32:131-140; Russell *et al.* (1982) *Gene* 20:231-243; Hallewell *et al.* (1985) *Nucleic Acids Res.* 13:2017-2034; Yoakum *et al.* (1982) *Proc. Natl. Acad. Sci. USA* 82: 1766-1770; Queen (1983) *J. Mol. Appl. Genet.* 2:1-10; De la Torre *et al.* (1984) *J. Biol. Chem.* 259:11571-11575; Shirakawa *et al.* (1984) *Gene* 28:127-132; Miyake *et al.* (1985) *J. Biochem.* 97:1429-1436; Studier *et al.* (1986) *J. Mol. Biol.* 189:113-130; Johnston *et al.* (1985) *Gene* 34:137-145; Nieto *et al.* (1997) *J. Biol. Chem.* 272:30860-30865; and Sizemore *et al.* (1993) *FEMS Microbiol. Lett.* 107:303-306. For general reviews, see Bauerle (ed.) "Inducible Gene Expression" Birkhauser, Boston, 1985; A. Smith (ed.) "Gene expression in recombinant microorganisms," M. Dekker, New York, 1994; Makrides (1996) *Microbiol. Rev.*

60:512-538; and de Vos *et al.* (1997) *Curr. Opin. Biotechnol.* 8:547-553. Most of the above-mentioned systems are capable of overexpression of one or more cloned genes. These systems often exhibit moderate-to-high basal expression levels, above which overexpression can be induced by manipulation of environmental conditions and/or provision of inducing molecules. Fusions between the P_{BAD} promoter of the arabinose operon and a heterologous gene have been used for the overexpression of heterologous genes. *See* U.S. Patent No. 5,028,530. However, in contrast to other systems, the P_{BAD} promoter can be extremely tightly regulated to provide very low basal levels of expression. *See*, for example, Guzman *et al.* (1995) *J. Bacteriology* 177:4121-4130. Construction of arabinose-dependent strains, generated by placing an essential gene under the control of *ara* regulatory elements, has been described. *See*, for example, Brown *et al.* (1995) *J. Bacteriol.* 177:4194-4197; Dalbey *et al.* (1985) *J. Biol. Chem.* 260:15925-15931; and Guzman *et al.*, *supra*.

Systems for drug screening have been described wherein overexpression of a target gene product results in acquisition of resistance to an inhibitor, identifying the gene product as a potential target of the inhibitor. *See*, for example, del Castillo *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:8860-8864. Screening for inhibitors of a particular enzyme has been accomplished by comparing the effect of a test compound on a strain that is defective for the enzyme with the effect of the test compound on a strain harboring a different mutation. *See*, for example, EP 644268. Other screening systems have been developed which depend on generating strains which express mutant proteins (e.g., temperature-sensitive proteins) and assessing their sensitivity to test compounds. *See*, for example, PCT Publication WO 96/23075.

Accordingly, highly-inducible regulatory systems with low basal expression levels would be extremely useful for the identification of essential genes and inhibitors of essential genes in microorganisms.

DISCLOSURE OF THE INVENTION

One object of the invention is to provide methods and compositions for identifying compounds which inhibit the growth or viability of an organism, regardless of whether the mechanism of action of the inhibitor and/or the function of the inhibitor's target is known. Another object of the invention is to provide compositions and methods for screening compounds using cells that are hypersusceptible to an inhibitor. An additional object of the invention is to provide methods for generating cells that are hypersusceptible to a known inhibitor, utilizing techniques of molecular genetics and recombinant DNA, in particular, techniques that permit regulated expression of a target gene, including underexpression, expression at normal levels, and overexpression. A further object of the invention is to provide compositions and methods for identifying essential genes and gene products of microorganisms, as well as genes and gene products that are involved in virulence and drug resistance. Yet another object of the invention is to provide methods and compositions for determining the mechanism of action of an inhibitor. A further object of the invention is to provide methods and compositions for controlled gene expression. An additional object is to provide methods and compositions that will allow expression of a particular target gene to be regulated at levels that are both lower and higher than those normally present in the cell.

Accordingly, in one aspect the invention provides cells in which the expression of a gene product involved in an essential function can be regulated. In particular, the invention provides cells in which the expression of a gene can be down-regulated to express the gene product below wild-type levels, as well as cells in which gene expression can be up-regulated to levels that are higher than wild-type. In some cases, expression of a gene product at lower-than-normal levels will, in and of itself, result in an impairment or absence of growth which defines the gene product as being essential. In other cases, environmental conditions (such as, for example, temperature, pH, nutrient sources, ionic strength, presence of other organisms, infection and/or presence of a compound) under

which expression of a particular level of a given gene product is essential can be determined.

In one aspect, the methods and compositions of the invention will allow expression of any gene in a cell to be independently regulated by external stimuli, such as nutrient concentration. Genes whose expression can be down-regulated to a point at which levels of that particular gene product become limiting for growth or other important cell function (*e.g.*, pathogenesis or resistance to antibiotics) can then be identified. Once such a gene has been identified, cells expressing that gene at any level between that which is limiting and any higher expression level can be challenged with a test compound. Compounds which exhibit higher potency against cells expressing lower levels of gene product are candidate inhibitors. It can be seen that, since this method depends simply on regulating levels of a particular gene product, it is not necessary beforehand to know the function of the gene product that is being regulated, nor is it necessary to know the mechanism of action of the inhibitor. Accordingly, knowledge of target function is not necessary for the identification of an inhibitor in the practice of the invention.

In another aspect, the invention provides methods and compositions for the identification of compounds that affect essential cellular processes, by exposing to a test compound cells in which expression of a gene product that is involved in an essential cellular process is regulated to a lower-than-normal level.

In yet another aspect, the invention provides methods and compositions for determining the target and mechanism of action of an inhibitor by exposing, to a test compound, cells in which expression of a gene product that is involved in an essential cellular process is regulated to a lower-than-normal level.

In yet another aspect, the invention provides methods and compositions for determining the target and mechanism of action of an inhibitor by exposing, to a test compound, a library of cells in which expression of a variety of gene products that are involved essential cellular processes are regulated to lower-than-normal levels.

In a further aspect, the practice of the invention will allow identification of genes encoding drug targets, genes encoding essential cellular functions, genes encoding virulence factors, genes encoding antibiotic resistance factors, polypeptides or fragments thereof that serve as drug targets or virulence factors; 5 polypeptides or fragments thereof that participate in essential cellular functions or antibiotic resistance; RNAs that serve as drug targets or virulence factors and RNAs that participate in essential cellular functions or antibiotic resistance.

Cells in which expression of a gene product that is involved in an essential cellular process is regulated to a lower-than-normal level can be obtained through 10 techniques of microbial genetics and molecular biology. For example, fusion of a heterologous regulatory element to an essential gene places that gene under the control of the heterologous regulatory element. The heterologous regulatory element may intrinsically provide lower expression levels than the essential gene's normal regulatory system, or the heterologous regulatory element may be 15 capable of being down-regulated. In either case, expression of the essential gene at lower-than-normal levels is possible.

The invention also provides methods and compositions for regulated gene expression, whereby expression is controlled over a range of levels ranging from underexpression through normal expression levels through overexpression.

20 Exemplary compositions include regulatable promoters, enhancers, operators and other transcriptional and/or translational control elements. Exemplary methods include methods for placing regulatable promoters, enhancers, operators and other transcriptional and/or translational control elements into operative linkage with a gene or coding sequence, and expression of such constructs in a cell, wherein 25 expression is regulated by an inducer and/or repressor.

Methods and compositions for regulated expression of a gene in a microorganism are also provided; wherein the methods utilize a construct comprising a gene, or a fragment thereof, in operative linkage with a regulatory element such as the *E. coli* P_{BAD} promoter or the P_{AGA} promoter of *S. pneumoniae*.

30 The methods comprise introducing the construct into a host cell, culturing the host cell in a growth medium and adjusting the concentration of one or more

modulator substances in the growth medium. Modulator substances can be inducers and/or negative modulators (*i.e.*, repressors) of the *raf* regulatory element(s) present in the construct.

5 In another embodiment, compositions and methods for making a construct, comprising a gene or a fragment thereof in operative linkage with a component of the *raf* regulatory region of *S. pneumoniae*, are provided. Such constructs can be chromosomal or extrachromosomal.

The invention will therefore be useful for drug screening, target identification, determining mechanisms of action of antibiotics, determining
10 mechanisms of virulence and antibiotic resistance, and for other purposes as will be apparent to those of skill in the art.

BRIEF DESCRIPTION OF THE DRAWINGS

15 **Figure 1** shows the nucleotide sequence of the *S. pneumoniae* *Px* and *Pm* regions. (SEQ ID NO: 1). The mutation in the repressor binding site of the *Px* promoter, converting a GGA sequence to GCG (mutant construction described in Example 6) is indicated on the bottom line of the figure.

Figure 2 is a schematic diagram of the *S. pneumoniae raf* gene cluster, including the two *raf* operons, with open reading frames (ORFs) represented by
20 arrows. Locations of promoters is also indicated. Shown for comparison is a schematic diagram of ORFs in the *msm* region of *S. mutans*.

Figure 3 shows the nucleotide sequence of the *raf* region of *S. pneumoniae* strain VSPN3026 (SEQ ID NO. 2). The general location of the *P_{AGA}* promoter is indicated by underlining.

25 **Figure 4** shows idealized results of an experiment in which the minimum inhibitory concentration of a compound is determined as a function of inducer concentration, in a cell in which target expression level is regulated by inducer concentration and the target is a single component which is inhibited by the compound.

30 **Figure 5** shows idealized results of an experiment in which the minimum inhibitory concentration of a compound is determined as a function of inducer

concentration, in a cell in which either 1) the target comprises multiple components and the compound interacts with a site defined by two or more of the components or 2) the compound interacts with multiple targets, and the level of one of the components (or targets) is regulated by inducer concentration.

5 **Figure 6** shows a scheme for replacement of wild type *murA* regulatory elements with an *ara* regulatory cassette.

Figure 7 shows growth of a P_{BAD} -*murA* fusion strain (*E. coli* VECO2055) as a function of arabinose concentration.

10 **Figure 8** shows the minimum inhibitory concentration of fosfomycin, as a function of arabinose concentration, for the P_{BAD} -*murA* fusion strain *E. coli* VECO2055, compared to wild-type.

Figure 9 shows minimum inhibitory concentrations of fosfomycin, ciprofloxacin, and tetracycline for the *E. coli* P_{BAD} -*murA* fusion strain VECO2055, expressed as a function of arabinose concentration.

15 **Figure 10** shows optical density measurements, at various times after inoculation, of cultures of VSPN3041 grown at different raffinose concentrations.

Figure 11 shows optical density measurements of the growth of VSPN3041 on either sucrose or raffinose.

20 **Figure 12** shows the growth of VSPN3041 and the parent isogenic strain VSPN3026, at different raffinose concentrations. Growth was measured by optical density after 10 hours of culture.

25 **Figure 13** shows the susceptibility of VECO2065, an *E. coli* strain having a chromosomal P_{BAD} -*def* fusion, to VRC483, an inhibitor of the *def* gene product. Susceptibility is presented as minimum inhibitory concentration (MIC) of VRC483 in $\mu\text{g/ml}$, as a function of inducer (arabinose) concentration. Also shown is the susceptibility of VECO2065 to fosfomycin and ciprofloxacin.

30 **Figure 14** shows the susceptibility of VECO2079, an *E. coli* strain having a chromosomal P_{BAD} -*folA* fusion, to trimethoprim, an inhibitor of the *folA* gene product. Susceptibility is presented as minimum inhibitory concentration (MIC) of trimethoprim in $\mu\text{g/ml}$, as a function of inducer (arabinose) concentration. Also shown is the susceptibility of VECO2079 to fosfomycin and ciprofloxacin, and

the susceptibility of the parent strain, VECO2054 (indicated by wt), to trimethoprim.

5 **Figure 15** shows the susceptibility of VECO2083, an *E. coli* strain having a chromosomal P_{BAD} -*gyrB* fusion, to novobiocin, an inhibitor of the *gyrB* gene product. Susceptibility is presented as minimum inhibitory concentration (MIC) of novobiocin in $\mu\text{g/ml}$, as a function of inducer (arabinose) concentration. Also shown is the susceptibility of VECO2083 to fosfomycin and ciprofloxacin.

10 **Figure 16** shows the susceptibility of VECO2068, an *E. coli* strain having a chromosomal P_{BAD} -*def* fusion and a *tolC* deletion, to VRC483, an inhibitor of the *def* gene product (indicated by the curve labeled VRC483). Susceptibility is presented as minimum inhibitory concentration (MIC) of VRC483 in $\mu\text{g/ml}$, as a function of inducer (arabinose) concentration. Also shown is the susceptibility of VECO2068 to fosfomycin and ciprofloxacin, and the susceptibility to VRC483 of the parent strain, VECO2066 (indicated by the curve labeled VRC483, *tolC*).

15 **Figure 17** shows the susceptibility of VSPN3044 to VRC483, an inhibitor of the *def* gene product. VSPN3044 contains a P_{AGA} -*def* transcriptional fusion, so that expression of the *def* gene product is regulated by raffinose. Susceptibility is presented as minimum inhibitory concentration (MIC) of VRC483 in $\mu\text{g/ml}$, as a function of inducer (raffinose) concentration. Also shown in the susceptibility of VSPN3044 to erythromycin and vancomycin, and the susceptibility of the parent strain VSPN3026 (indicated by VRC483wt) to VRC483.

20 **Figure 18** shows the susceptibility of VECO2524 (P_{BAD} -*lpxC*, ΔtolC) to L159692, an antibacterial compound that targets the *lpxC* gene product. Minimum inhibitory concentration is shown as a function of arabinose concentration. Also shown are minimum inhibitory concentrations of linezolid and erythromycin as a function of arabinose concentration.

MODES FOR CARRYING OUT THE INVENTION

30 The practice of the present invention will employ, unless otherwise indicated, conventional techniques in organic chemistry, biochemistry, molecular biology, microbiology, genetics, recombinant DNA, and related fields as are

within the skill of the art. These techniques are fully explained in the literature. See, for example, Maniatis, Fritsch & Sambrook, MOLECULAR CLONING: A LABORATORY MANUAL, Cold Spring Harbor Laboratory Press (1982); Sambrook, Fritsch & Maniatis, MOLECULAR CLONING: A LABORATORY MANUAL, 5 Second Edition, Cold Spring Harbor Laboratory Press (1989); Ausubel, *et al.*, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley & Sons (1987, 1988, 1989, 1990, 1991, 1992, 1993, 1994, 1995, 1996); Silhavy *et al.*, EXPERIMENTS WITH GENE FUSIONS, Cold Spring Harbor Laboratory Press (1984); Gerhardt *et al.*, METHODS FOR GENERAL AND MOLECULAR MICROBIOLOGY, American 10 Society for Microbiology, Washington, D.C., 1994; Lorian, ANTIBIOTICS IN LABORATORY MEDICINE, 4th ed., Williams & Wilkins, Baltimore, 1996; and Murray *et al.* MANUAL OF CLINICAL MICROBIOLOGY, 6th ed., American Society for Microbiology, Washington, D.C., 1995.

All patents, patent applications and publications cited herein are 15 incorporated by reference in their entirety.

The present invention provides methods and compositions useful for identification of compounds that affect an essential cellular process, compounds that interfere with mechanisms of resistance, and compounds that interfere with virulence factors; for identification of the target or targets of a compound that 20 affects an essential cellular process, a mechanism of resistance or a virulence factor; for identification of a gene or genes encoding a target or targets of a compound that affects an essential cellular process, a mechanism of resistance or a virulence factor; and for identification of genes, RNAs and polypeptides involved in essential cellular processes, mechanisms of resistance or virulence. 25 Identification is facilitated by controlled expression of a gene that is involved in an essential cellular process. Knowledge of the function of a gene or its product is not required, either to identify it as being involved in an essential cellular process, or to identify a compound which affects the gene product.

Cell types

In one embodiment, the present invention will be used in the identification of compounds which have activity against microorganisms. Accordingly, compositions embodied by the invention will include microorganisms wherein the expression of an essential gene of the microorganism is regulated by fusion to a heterologous regulatory element. Similarly, target genes and polypeptides whose expression is regulated by a heterologous regulatory element will often be those that are essential for viability of a microorganism, or responsible for its virulence or drug resistance.

Microorganisms can be either prokaryotic or eukaryotic; and prokaryotes can be either Gram-positive or Gram-negative. Exemplary prokaryotes include, but are not limited to: *Staphylococcus* (e.g., *S. aureus*, *S. epidermidis*), *Streptococcus* (e.g., *S. pneumoniae*, *S. pyogenes*, *S. agalactiae*), *Enterococcus* (*E. faecalis*, *E. faecium*), *Neisseria*, *Branhamella*, *Listeria*, *Bacillus* (e.g., *B. subtilis*), *Corynebacterium*, *Erysipelothrix*, *Gardnerella*, *Nocardia*, *Mycobacterium*, enterobacteriaceae, *Escherichia* (e.g., *E. coli*), *Salmonella*, *Shigella*, *Yersinia*, *Enterobacter* (e.g., *E. cloacae*), *Klebsiella* (e.g., *K. pneumoniae*, *K. oxytoca*), *Citrobacter*, *Serratia*, *Providencia*, *Proteus* (e.g., *P. mirabilis*, *P. vulgaris*), *Morganella* (e.g., *M. morganii*), *Edwardsiella*, *Erwinia*, *Vibrio*, *Aeromonas*, *Helicobacter* (e.g., *H. pylori*), *Campylobacter*, *Eikenella*, *Pasteurella*, *Pseudomonas* (e.g., *P. aeruginosa*), *Burkholderia*, *Stenotrophomonas*, *Acinetobacter*, *Ralstonia*, *Alcaligenes*, *Moraxella*, *Legionella*, *Francisella*, *Brucella*, *Haemophilus* (e.g., *H. influenzae*), *Bordetella*, *Clostridium*, *Bacteroides*, *Porphyromonas*, *Prevotella*, *Fusobacterium*, *Borrelia*, *Chlamydia*, *Rickettsia*, *Ehrlichia* and *Bartonella*.

Exemplary eukaryotic microorganisms include, but are not limited to, yeasts and fungi, for example, *Candida* (e.g., *C. albicans*), *Cryptococcus*, *Pneumocystis*, *Histoplasma*, *Blastomyces*, *Coccidioides*, *Aspergillus*, *Fusarium*, *Saccharomyces* and *Schizosaccharomyces*.

The practice of the invention can also be applied to eukaryotic cells, such as plant cells, mammalian cells and human cells. In one embodiment, malignant

cells which are resistant to a therapeutic can be analyzed to determine the locus of resistance and to identify compounds that will reverse resistance by interacting with the cellular component responsible for resistance. In this context, a therapeutic can comprise a compound, such as a drug, a composition comprising multiple compounds, or a physical treatment, such as radiation.

Essential cellular functions

In one embodiment, the invention provides methods and compositions for identifying genes and/or gene products involved in essential cellular functions.

10 An essential function for a particular cell will depend on the genotype of the cell and the cell's environment. By way of example, essential cellular functions are those which are involved in replication, repair, recombination and transcription of genetic material; protein synthesis (translation), processing and transport; protein export; anabolic synthesis of cellular molecules; catabolism of cellular nutrients;

15 synthesis of cell membranes and cell walls; lipid metabolism; protein metabolism; energy metabolism; cell division; cell shape; filamentation; regulation; DNA binding; RNA binding; efflux systems; transport systems; virulence or pathogenicity; and drug resistance. Protein metabolism can include protein modifications such as glycosylation, phosphorylation, acetylation and

20 ubiquitination, to name but a few examples. Gene products that can be involved in essential cellular processes include, but are not limited to, topoisomerases, nucleases, recombinases, primases, helicases, DNA polymerases, RNA polymerases, histone modifying enzymes, kinases, phosphatases, acetylases, deacetylases, formylases, deformylases, chaperonins, ion transporters,

25 cytoskeletal elements, colicins, cytochromes, ribosomal proteins, transfer RNAs, ribosomal RNAs, hydrolases, proteases, epimerases, rotamases, synthases, racemases, dehydrogenases, transferases, ligases, reductases, oxidases, transglycosylases, transpeptidases, peptidases, GTPases, ATPases, translocases, ribonucleases, transcription factors, sigma factors, ribosomal release factors,

30 structural RNAs and structural proteins.

More generally, an essential cellular process is any process which, when it occurs at a lower rate or to a lesser extent than normal, negatively influences the viability of the cell. Methods for determination of cell viability are well-known to those of skill in the art and include, but are not limited to, vital staining, cell counting, either microscopically or by colony counting following serial dilution and plating of cell cultures, measurement of light scattering by cell cultures, fluorescence-activated cell sorting, incorporation of polynucleotide and/or polypeptide precursors, reporter gene expression, and measurement of cell weight and/or volume.

The types of molecules that can participate in essential cellular processes can include nucleic acids, polypeptides and other cellular macromolecules. Nucleic acids will include, for example, DNA; regulatory RNA molecules, such as ribozymes and antisense RNA; transfer RNA and ribosomal RNA. Polypeptides can include, for example, structural proteins, enzymes, receptors, intracellular signaling molecules, and cellular adhesion molecules.

Regulatory elements

In one aspect of the invention, the expression of a gene involved in an essential cellular function is regulated by fusion of the gene, or a fragment thereof, to a heterologous regulatory element. A heterologous regulatory element is one that is not normally associated with, and does not normally regulate, the gene which it regulates in the practice of the invention. Regulatory elements can comprise transcriptional, post-transcriptional, translational, and post-translational elements; as well as regulatory elements related to replication. By way of example, transcriptional regulatory elements can include promoters, enhancers, operators, and elements that modulate the rate of transcription initiation, elongation and/or termination; post-transcriptional regulatory elements can include those influencing messenger stability, processing and transport; translational regulatory elements can include those which modulate the frequency of translation initiation and the rate of translational elongation; post-translational regulatory elements can include those which influence protein processing,

stability and transport; and replication-associated regulatory elements can include those related to gene dosage.

In preferred embodiments, the heterologous regulatory element comprises a regulatable promoter. In a particularly preferred embodiment, the regulatable promoter is the *araBAD* promoter, also known as P_{BAD} . Regulation by P_{BAD} has been the subject of extensive study and its regulatory properties are well-understood. See, for example, Schleif (1992) *Ann. Rev. Biochem.* **61**:199-223; Guzman *et al.* (1995) *J. Bacteriology* **177**:4121-4130; and Gallegos *et al.* (1997) *Microbiology and Molecular Biology Reviews* **61**:393-410.

The P_{BAD} promoter is regulated by the AraC protein, which has both positive and negative regulatory activities. In the absence of L-arabinose or other inducers (such as, for example, L-ribose), AraC represses transcription from P_{BAD} by binding to sites upstream of the P_{BAD} transcription initiation site. Inducers such as L-arabinose interact with the AraC protein to form an activator of P_{BAD} transcription that binds to different upstream sites to stimulate transcription. With respect to the present invention, desirable features of the AraC/ P_{BAD} regulatory system are the very low basal levels of transcription obtained in the absence of arabinose and the direct relationship between transcription from P_{BAD} and the concentration of arabinose in the medium. See, for example, Guzman *et al.*, *supra*.

The activity of P_{BAD} is directly proportional to the concentration of arabinose in the environment and, importantly, at low arabinose concentration, very low basal levels of expression are obtained. The P_{BAD} promoter is also subject to regulation by catabolite repression, mediated by cyclic AMP and by the cyclic AMP receptor protein, also known as the catabolite repressor protein (CRP). Thus, further modulation of P_{BAD} expression can be obtained by regulating the concentration of glucose (or other carbon source such as, for example, glucose-6-phosphate) in the environment, which modulates CRP activity within the cell. In particular, minimal expression of P_{BAD} (maximal repression) is obtained in the presence of glucose and the absence of arabinose. Withdrawal of glucose from the medium and addition of arabinose (or another inducer) results in

rapid induction of transcription from P_{BAD} wherein the expression level is proportional to the arabinose concentration. Expression levels varying over a 1,000-fold range can be obtained, depending on the inducer concentration. See, for example, Guzman *et al.*, *supra*.

5 The P_{BAD} promoter or any other promoter of the AraC/XylS family, from any prokaryotic or eukaryotic organism, can be used in the practice of the invention. See, for example, Gallegos *et al.*, *supra*; de Vos *et al.* (1997) *Curr. Opin. Biotechnol.* 8:547-553; and Kleerebezem *et al.* (1997) *Mol. Microbiol.* 24:895-904. Particularly preferred are the P_{BAD} promoters of *E. coli* and *S.*
10 *typhimurium*.

Another regulatory system that is useful in the practice of the invention is the *malM/malX* system of *S. pneumoniae*, regulated by MalR. MalR is a repressor that controls the expression of the maltosaccharide regulon in *S. pneumoniae* and belongs to the LacI-GalR family of repressors. Two operons are
15 regulated in opposite direction, *malXCD* (P_x promoter) and *malMP* (P_m promoter), see Figure 1 (SEQ ID NO: 1). Stassi *et al.* (1982) *Gene* 20:359-366; and Nieto *et al.* (1997) *J. Biol. Chem.* 272:30860-30865. Affinity of MalR for P_m is higher than for P_x and, in both cases, a high basal level of expression has been reported. Nieto *et al.* (1997) *J. Biol. Chem.* 272:30860-30865. Example 6,
20 *infra*, describes fusion of *mal Px* to a catalase gene and modification of the *mal Px* promoter to obtain tight regulation by maltose in minimal medium.

Yet another example of a regulatory system that is useful in the practice of the invention is the *raf* regulatory system of *Streptococcus pneumoniae*. Example 14 shows that the *rafR* gene product acts as a positive regulator of promoters such
25 as P_{AGA} , the promoter for the *S. pneumoniae* α -galactosidase gene. Thus, fusion of a target gene to P_{AGA} , in a cell expressing *rafR* function, will allow raffinose-regulated expression of the target gene. See Example 7. Additional regulatory elements in the *raf* regulatory system include the promoter of the *rafR* gene, P_{rafR} , and the promoter of the *rafE* gene, P_{rafE} .

30 The P_{AGA} promoter was discovered through a search of the *S. pneumoniae* genome sequence, disclosed at <http://www.tigr.org>. The sequence was searched

for sequences that might encode homologues to the AraC/XylS family of transcriptional activators. Gallegos *et al.* (1997) *Microbiol. Molec. Biol. Reviews* 61:393-410. An open reading frame (ORF) encoding a protein homologous to the *Streptococcus mutans msmR* gene was identified and named *rafR*. The organization of additional ORFs in the vicinity of *rafR* was also investigated. As a result of these investigations, a gene cluster was identified, comprising two ORFs encoding regulatory proteins, *rafR* and *rafS*, an intergenic region, and six ORFs encoding structural proteins. See Figure 2. The gene cluster contains two operons transcribed divergently: a regulatory operon encoding *rafR* and *rafS*, and a metabolic operon which encodes *aga*, *rafE*, *rafF*, *rafG*, *gtfA* and possibly *rafH*.

The nucleotide sequence of the region of the *raf* gene cluster encompassing *rafS*, *rafR*, and *aga*, in *S. pneumoniae* strain VSPN3026 was determined. See Figure 3 (SEQ ID NO: 2). The *rafS* gene was determined to lie between the complements of nucleotide coordinates 1001-291 of this sequence, with the region encoding RafS protein lying between the complements of nucleotides 938-294. The *rafR* gene was determined to lie between the complements of nucleotides 1798-935, with the RafR coding region complementary to nucleotides 1795-938. The *aga* gene extended from nucleotides 1903-4065, with the coding region lying between 1903-4062. Several differences between the *raf* sequences of VSPN3026 (Figure 3) and those disclosed in the database at <http://www.tigr.org> were detected. These differences, presented in Table 1, are likely to represent polymorphisms between different strains of *S. pneumoniae*.

Table 1: Unique sequences in the *S. pneumoniae raf* region of VSPN 3026

Position in VSPN3026 sequence (SEQ ID NO: 2)	SEQUENCE	
	VSPN 3026	database
326-329	ATCC	ATACC
441	A	G
561	T	A
633	A	G
765	T	C
794	T	C
828	A	G
842	A	C
953	C	T
997	A	C
1490	A	G
1513	A	C
1665	C	G
1760	A	G
1792	G	A
2157	T	C
2739	C	T
2844	T	G
3191-3192	AT	GC
3287	C	T
3297	G	T
3399	A	G
3405	C	T
3495	A	G
3662	A	T
3693	G	A
3818	C	T

The *S. pneumoniae raf* gene cluster is organized into two domains. One domain includes the two regulatory ORFs *rafR* and *rafS*, and the other includes genes that are probably involved in uptake and catabolism, based on their

homology to *S. mutans* genes. See Figure 2. The term “*raf* gene cluster” refers to the *raf* transcriptional units and their related regulatory genes, in particular the region of the *S. pneumoniae* genome comprising the *rafR*, *rafS*, *aga*, *rafE*, *rafF*, *rafG*, *gtfA* and *rafH* genes, as well as the intergenic regions associated with these genes. Intergenic regions refer to DNA sequences which do not encode protein, but which lie adjacent to protein-coding regions of DNA sequence. Intergenic regions will often contain regulatory sequences such as promoters and operators, although regulatory sequences can also be located in coding regions.

Directly upstream of *rafR* is a divergently-transcribed gene, *aga*, with sequence homology to *S. mutans* α -galactosidase. Construction of a strain with a mutation in the *aga* region, followed by α -galactosidase assay of the mutant strain, shows that the *S. pneumoniae* *aga* gene does indeed encode a polypeptide with α -galactosidase activity. See Example 14. Downstream of *aga* are additional genes encoding proteins homologous to the *msm* transport system, and a gene called *gtfA*, which is a homologue of *S. mutans* sucrose phosphorylase. Although it contains several homologous ORFs, the fact that the *S. pneumoniae* *raf* gene cluster contains two regulatory genes suggests that its regulation may be more complex than that of the *msm* gene cluster in *S. mutans*.

The *S. pneumoniae* *raf* gene cluster contains at least two regulatory genes, *rafR* and *rafS*, an intergenic region, and at least five structural genes: *aga*, *rafE*, *rafF*, *rafG* and *gtfA*. See Figure 2. Sequences which regulate the expression of the regulatory and structural genes of the *S. pneumoniae* *raf* gene cluster are likely to be found in the intergenic region and within genes adjacent to the intergenic region. Such sequences are denoted *raf* regulatory sequences and include, for instance, promoter and operator sequences, such as the *rafR* promoter (P_{rafR}), the α -galactosidase promoter (P_{AGA}) and the *rafE* promoter P_{rafE} . Promoter sequences are those to which RNA polymerase binds to initiate transcription. Operator sequences are those to which regulatory proteins (such as, for example, activators and repressors) bind, thereby influencing the ability of RNA polymerase to bind to the promoter. In general, repressors inhibit binding of RNA polymerase, and activators facilitate binding (or relieve repressor-mediated inhibition). Transcript

analysis by RT-PCR has provided results consistent with the locations of the P_{AGA} , P_{rafE} and P_{rafR} promoters being as shown in Figure 2. Based on the presence of sequence elements homologous to well-known prokaryotic transcriptional regulatory sequences, the location of P_{AGA} was determined to be between
5 nucleotides 1796-1902 of the sequence presented in Figure 3. The P_{rafR} promoter is also believed to lie within this region.

In one embodiment, the invention provides sequences from the *raf* regulatory region, such as *raf* promoter and operator sequences, for the regulated expression of coding sequences, which can include, for example, homologous and
10 heterologous genes or gene fragments. With respect to the *S. pneumoniae* regulatory sequences disclosed herein, a homologous gene is one that is normally found in association with the regulatory sequences in nature. A heterologous sequence, by contrast, is a sequence from *S. pneumoniae* or any other organism, that is not normally found in association with *S. pneumoniae raf* regulatory
15 sequences in nature. Exemplary *S. pneumoniae* regulatory sequences include, but are not limited to, the *rafR* promoter (P_{rafR}), the α -galactosidase promoter (P_{AGA}), and the promoter of the *rafE* gene, P_{rafE} .

As discussed *supra* with respect to sequence homology, and demonstrated experimentally in Example 14 *infra*, the *rafR* gene product acts as a positive
20 regulator and the *rafS* gene product acts as a negative regulator of the *raf* operons. Growth of cells in the presence of raffinose induces expression of genes under the control of *raf* regulatory sequences, while growth of cells on sugars other than raffinose inhibits expression of genes under the control of *raf* regulatory sequences. Consequently, the methods and compositions provided by the
25 invention allow for both overexpression and underexpression of a gene, mediated by *raf* regulatory sequences. The basal level of expression is low and the range of expression level between repressed (cells grown on maltose, for example) and induced (cells grown on raffinose) conditions is approximately a thousand-fold. See Example 14, *infra*.

30 In one embodiment, the invention provides recombinant constructs for regulation of expression of a gene of interest. The recombinant constructs are

made using standard methods of molecular biology and biotechnology to place a coding sequence in operative linkage with *raf* regulatory region sequences, either by insertion of a coding sequence in proximity to a *raf* regulatory sequence, or by insertion of a *raf* regulatory sequence in proximity to coding sequence. In preferred embodiments, the *raf* regulatory sequence will be upstream of the coding sequence when they are placed in operative linkage. Locations of restriction enzyme recognition sequences within the *raf* gene cluster, for use as insertion sites, can be easily determined by one of skill in the art from the nucleotide sequence of the *raf* gene cluster. Alternatively, various *in vitro* techniques can be used for insertion of a restriction enzyme recognition sequence at a particular site, or for insertion of heterologous sequences at a site that does not contain a restriction enzyme recognition sequence. Such methods include, but are not limited to, oligonucleotide-mediated heteroduplex formation for insertion of one or more restriction enzyme recognition sequences (*see*, for example, Zoller *et al.* (1982) *Nucleic Acids Res.* 10:6487-6500; Brennan *et al.* (1990) *Roux's Arch. Dev. Biol.* 199:89-96; and Kunkel *et al.* (1987) *Meth. Enzymology* 154:367-382) and PCR-mediated methods for insertion of longer sequences. *See*, for example, Zheng *et al.* (1994) *Virus Research* 31:163-186.

Operative linkage refers to an arrangement of one or more regulatory sequences with one or more coding sequences, such that the regulatory sequence(s) is capable of exerting its regulatory effect on the coding sequence. By way of illustration, a transcriptional regulatory sequence or a promoter is operably linked to a coding sequence if the transcriptional regulatory sequence or promoter promotes transcription of the coding sequence. Similarly, an operator is considered operatively linked to a promoter or to a coding sequence if binding of a repressor to the operator inhibits initiation at the promoter so as to prevent or diminish expression of the coding sequence. An operably linked transcriptional regulatory sequence is generally joined in *cis* with the coding sequence, but it is not necessarily directly adjacent to it.

Recombinant constructs comprising coding sequences in operative linkage with one or more *raf* regulatory region sequences can also comprise other types of

sequence including, but not limited to, replication origins, selectable markers (including, but not limited to, those encoding antibiotic resistance), transcription termination sites, sequences specifying translation initiation and termination, sequences mediating mRNA processing and/or stability and multiple cloning sites.

- 5 In preferred embodiments, these additional sequences are functional in Gram-positive microorganism, such as, for example, *Streptococci*, *Staphylococci*, *Enterococci*, and *Lactococci*. Preferred species include, for example, *S. pneumoniae*, *S. pyogenes*, *S. agalactiae*, Lancefield group A streptococci, Lancefield group B streptococci, Lancefield group C streptococci, Lancefield
10 group F streptococci, Lancefield group G streptococci, and viridans streptococci. Preferred non-streptococcal species in which these additional sequences are functional include, for example, enterococci such as *E. faecalis*, and *E. faecium*, and lactococci such as *L. lactis*. Methods for the construction of such recombinant constructs are well-known to those of skill in the art. See, for
15 example, Sambrook *et al.*, *supra*. It will also often be useful to include a selectable marker in the recombinant construct, to aid in the isolation and identification of cells comprising the construct. Selectable markers include those which facilitate positive selection, such as a sequence which encodes antibiotic resistance, and those which facilitate negative selection. Bochner *et al.* (1980) *J.*
20 *Bacteriol.* 143:926-933; and Gay *et al.* (1985) *J. Bacteriol.* 164:918-921. Recombinant constructs can exist as freely-replicating extrachromosomal elements, such as plasmids or episomes, or can exist as chromosomal recombinants, such as would be achieved either by integration of a *raf* regulatory cassette into the chromosome of a microorganism adjacent to a gene of interest, or
25 by insertion of a gene of interest into the chromosome adjacent to a *raf* regulatory sequence, for example. Methods for obtaining chromosomal integration of recombinant constructs have been described, for example, by Gerhardt *et al.*, METHODS FOR GENERAL AND MOLECULAR MICROBIOLOGY, American Society for Microbiology, Washington, D.C., 1994; Link *et al.* (1997) *J. Bacteriol.*
30 179:6228-6237; and Metcalf *et al.* (1996) *Plasmid* 35:1-13.

A coding sequence, as present in a recombinant construct, can encode a full-length gene product (*i.e.*, the length normally found in the wild-type cell) or any fragment of a gene product. A gene product can be a RNA or a polypeptide; untranslated RNA gene products can include structural, catalytic and regulatory RNA molecules. Examples of untranslated RNA gene products include, but are not limited to, tRNA, rRNA, antisense RNAs and ribozymes. In one embodiment, a coding sequence comprises a gene, which can encode a virulence factor, a resistance factor, or a gene product whose function is essential for a cell under a particular set of environmental conditions. Any gene of interest can be placed in operative linkage with *raf* regulatory region sequences, so that its expression is regulated by the *raf* regulatory region sequences.

In one embodiment, the invention provides recombinant constructs capable of regulating the expression of coding sequences in a host cell. These constructs comprise one or more *raf* regulatory sequences in operative linkage with a coding sequence. The constructs are suitable for use in any cell in which *raf* operon regulatory sequences are functional. Since the *raf* regulatory proteins RafR and RafS can be introduced into a cell along with, or as part of the above-mentioned recombinant construct, regulation of a coding sequence by a *raf* regulatory sequence will be attainable in many cells, which can include both Gram-positive and Gram-negative microorganisms. In preferred embodiments, the host cell is a Gram-positive microorganism, such as, for example, *Streptococci*, *Staphylococci*, *Enterococci*, and *Lactococci*. Preferred species include, for example, *S. pneumoniae*, *S. pyogenes*, *S. agalactiae*, Lancefield group A streptococci, Lancefield group B streptococci, Lancefield group C streptococci, Lancefield group F streptococci, Lancefield group G streptococci, and viridans streptococci. Preferred non-streptococcal species in which the *raf* regulatory system can be utilized for regulated expression of coding sequences include, for example, enterococci such as *E. faecalis*, and *E. faecium*, and lactococci such as *L. lactis*.

In the practice of one aspect of the invention, the recombinant construct is introduced into a host cell to provide regulated expression of a coding sequence.

Introduction of the construct into a host cell is performed by methods that are well-known to those of skill in the art, including, for example, natural or artificial transformation, transduction, conjugation, microinjection, transfection, electroporation, CaPO₄ co-precipitation, DEAE-dextran, lipid-mediated transfer, particle bombardment, *etc.*

Host cells are cultured in any suitable growth medium, including liquid or solid media. Appropriate growth media for various types of microorganisms are well-known to those of skill in the art. *See*, for example, Bergey's Manual of Systematic Bacteriology, vol. 2, Williams & Wilkins, Baltimore, 1980; Gerhardt *et al.* "Methods for General and Molecular Microbiology," American Society for Microbiology, Washington, D.C., 1994; and Murray *et al.*, *supra*.

Modulator substances can be added to the growth medium to influence the transcriptional activity of *raf* regulatory sequences. Such effects will be manifested as changes in the expression level of a coding sequence to which the *raf* regulatory sequences are operatively linked. The modulator substances can be generally characterized as inducers, which increase transcriptional activity, or negative modulators, which decrease transcription. In one embodiment of the invention, a modulator substance is a metabolite; in a preferred embodiment, it is a carbon source, in a more preferred embodiment, it is a sugar and, in a particularly preferred embodiment, raffinose serves as an inducer and maltose as a negative modulator.

The present invention utilizes systems which provide low basal expression levels and a high degree of induction. Such methods and compositions can be used, for example, to identify compounds which inhibit the growth of a microorganism, and for discovery of drug targets, including genes involved in virulence and drug resistance. Because the *S. pneumoniae raf* regulatory system is characterized by an induction level of at least 1,000-fold over a low basal expression level (see Example 14), it is well-suited for use in the practice of the invention.

Accordingly, *raf* regulatory sequences can be used to identify an essential gene of a microorganism, to regulate the level of expression of an essential gene,

and to identify inhibitors of essential genes and gene products, as disclosed herein. These features are accomplished by fusing *raf* regulatory sequences, such as *P_{rafR}*, *P_{rafE}*, *P_{AGA}* or others, to a homologous or heterologous coding sequence encoding an essential gene, such that the coding sequence is under the transcriptional
5 control of the *raf* regulatory sequences. Essential genes can include those which are essential for the growth of *S. pneumoniae*, or those which are essential for the growth of any other microorganism. The essentiality of a gene may depend on the *in vivo* or *in vitro* environment of the cell in which it is expressed. For example, in the presence of an effective concentration of an antibiotic, a gene encoding
10 resistance to that antibiotic is an essential gene. Example 7 shows the construction and properties of a fusion between the *S. pneumoniae* α -galactosidase promoter (*P_{AGA}*) and the *S. pneumoniae* leader peptidase (*spi*) gene. The fusion places the *spi* gene under raffinose control, limiting cell growth at low raffinose concentrations.

15 Additional positively-regulated promoter/activator systems will also find use in the practice of the invention. These include, but are not limited to, those for rhamnose utilization (regulated by the *rhaS* or *rhaR* gene products), melibiose utilization (regulated by the *melR* gene product), xylose utilization (regulated by the *xyIR* gene product), *p*-hydroxyphenylacetic acid utilization (regulated by the
20 *hpaA* gene product), and urease production (regulated by the *ureR* gene product). See Gallegos *et al.*, *supra*, for additional examples of positively regulated systems. Additional regulatable promoters that will be of use in the present invention will be well-known to those of skill in the art. They include, but are not limited to, *lac*, which is regulated by lactose and glucose; *trp*, which is regulated
25 by tryptophan, *tac*, which is regulated by lactose, *tet*, which is regulated by tetracycline and tetracycline analogues, *gal*, which is regulated by galactose, T7, which is regulated by provision of T7 RNA polymerase, T3, which is regulated by provision of T3 RNA polymerase, SP6, which is regulated by provision of SP6 RNA polymerase, λ _R, which is regulated by λ repressor (the *cI* gene product),
30 and λ _{PL}, which is regulated by λ repressor (the *cI* gene product). Additional promoters from Gram-negative organisms which can be tested for their degree of

regulatability and can be useful in the practice of the invention include, but are not limited to, *lpp*, *phoA*, *recA*, *proU*, *cst-I*, *tetA*, *cadA*, *nar*, *lpp-lac*, *cspA*, T7-*lac*, pL-T7, T3-*lac*, T5-*lac*, *nprM-lac*, VHb, promoters regulated by two-component regulatory systems, and promoters regulated by the *araC/XylS* family of
5 regulators. Two-component systems include those which utilize protein phosphorylation as a mechanism of signal transduction. In one embodiment, a sensor protein is phosphorylated upon receipt, by the cell, of an environmental stimulus. The phosphate group is then transferred to a regulator protein that undergoes a phosphorylation-induced conformational change which elicits a
10 response such as, for example, gene transcription. *See*, for example, Makrides, *supra*; J.A. Hoch and T.J. Silhavy, *eds.* (1995) "Two-Component Signal Transduction," American Society for Microbiology, Washington, D.C.; and Gallegos *et al.*, *supra*. Additional promoters from Gram-positive organisms which can be tested for their degree of regulatability and can be useful in the
15 practice of the invention include, but are not limited to, *spac-I*, *xylA*, *lacA*, *lacR*, P15, *dnaJ*, *sodA*, *prtP*, *prtM*, PA170, *trpE*, *nisA*, *nisF malX*, *malM*, *xyl*, and bacteriophage promoters from ϕ r11 and ϕ 31. *See*, for example, de Vos *et al.*, *supra*. Although some of these promoters are not capable, using current techniques, of basal expression levels as low as those that can be obtained with
20 P_{BAD}, they will find use in less-preferred embodiments of the invention.

Construction of gene fusions

In a preferred embodiment of the invention, fusion of a heterologous regulatory element to a gene encoding an essential cellular function is
25 accomplished by insertion of an *ara* regulatory cassette into the chromosome of the organism under study, or insertion of an *ara* regulatory cassette into a plasmid resident in the organism under study. The *ara* regulatory cassette can include a DNA molecule containing, in the following order, the *araC* gene, P_C (the *araC* promoter) and P_{BAD} (the promoter regulating expression of the *araB*, *araA*, and
30 *araD* genes). This is the order in which these elements are arranged on the *E. coli* and *S. typhimurium* chromosomes, in which the P_C and P_{BAD} promoters are

adjacent and oriented divergently. Insertion of this cassette will provide AraC function to the cell and place downstream coding sequences under the control of P_{BAD} , which is regulated by AraC. Alternatively, a cassette containing only P_{BAD} can be inserted, if AraC function is already provided by the cell.

5 In another embodiment, a cassette containing a gene or nucleotide sequence of interest can be inserted into the chromosome adjacent to P_{BAD} such that the gene or sequence comes under the transcriptional control of P_{BAD} . See Example 13. Furthermore, it will be apparent to one of skill in the art that a fusion between P_{BAD} (or any other regulatory element) and a gene or nucleotide
10 sequence of interest can itself be moved to any one of a number of different chromosomal or extrachromosomal locations, using techniques that are well-known in the art.

By way of example, fusions can be obtained by random insertion of an *ara* regulatory cassette into a chromosome or a plasmid of a microorganism, followed
15 by screening for strains dependent on arabinose for growth. Arabinose-dependent strains will be those in which sequences encoding an essential cellular function have been fused to the *ara* regulatory cassette in such a way that the coding sequences have come under P_{BAD} control. A coding sequence can encode a full-length gene product (*i.e.*, the length normally found in the wild-type cell) or any
20 fragment of a gene product capable of encoding an essential cellular function. A gene product can be a RNA or a polypeptide; untranslated RNA gene products can include structural, catalytic and regulatory RNA molecules.

Random chromosomal integration is typically achieved using transposons. Transposons are DNA segments which have the ability to insert randomly within
25 the a chromosome or plasmid of a host organism. Very little homology is required between the ends of a transposon and its integration site and the process is independent of the host's homologous recombination system. Transposon insertion is typically monitored by selection for an antibiotic resistance marker carried on the transposon. Because transposons can have low site specificity, they
30 are widely used for random inactivation by gene disruption.

Efficient targeted chromosomal integration of an exogenous sequence, involving site-specific recombination, typically requires a stretch of homology of 200 base pairs or more and utilizes the host's homologous recombination system to achieve integration. Targeted integration typically involves a recombination
5 event between the chromosome and a conditionally replication-defective plasmid containing chromosomal sequences and an antibiotic resistance marker. Under conditions that are non-permissive for plasmid replication, and in the presence of selective agent, the majority of surviving cells are those in which targeted recombination has occurred between the homologous sequences in the plasmid
10 and the chromosomal DNA. Gerhardt *et al.*, *supra*; Link *et al.* (1997) *J. Bacteriol.* 179:6228-6237; and Metcalf *et al.* (1996) *Plasmid* 35:1-13. The same considerations apply to targeted insertion within a plasmid.

By way of example, one method for generating a fusion of an *ara* regulatory cassette to a cellular coding sequence is by flanking the regulatory
15 cassette with sequences homologous to the targeted coding sequence, as described in Example 1, *infra*; however, other methods for generating gene fusions will be known to those of skill in the art. *See*, for example, Casadaban *et al. Meth. Enzymology*, vol. 100 (ed. R. Wu, L. Grossman, K. Moldave) Academic Press, New York, 1983) pp. 293-308; Silhavy *et al.*, *supra*; and Gerhardt *et al.*, *supra*.
20 Additional embodiments of the invention include extrachromosomal gene fusions residing, for example, on plasmids. Such plasmid fusions can be constructed *in vivo* or *in vitro*, using techniques of genetics and recombinant DNA which are well-known to those of skill in the art. *See*, for example, Sambrook *et al.*, *supra*; Ausubel, *et al.*, *supra*; Silhavy *et al.*, *supra*; and Gerhardt *et al.*, *supra*. For the
25 purposes of the present invention, nucleic acids constructed *in vitro* can be introduced into cells by methods that are well-known in the art, including transformation with naked DNA, electroporation, microinjection, calcium phosphate-mediated transfer, DEAE-dextran-mediated transfer, gene gun, *etc.*, to generate transformed cells.

30 It is clear that methods similar to those described above for *ara* regulatory cassettes can also be applied to the construction and integration of regulatory

cassettes comprising *mal*, *raf*, or other regulatory elements which allow controlled expression of a gene to which they are operatively linked.

Methods for controlled, low-level expression

5 In a preferred embodiment of the present invention, expression of an essential gene is regulated to a low basal level. A low basal level is less than 50% of wild-type, preferably, less than 30%, more preferably, less than 20%, and, most preferably, less than 10%. In some cases, expression of an essential gene at a low basal level will render a cell non-viable; in other cases, it will render a cell
10 hypersusceptible to a biologically-active agent. An example of, low, basal-level regulation by *ara* P_{BAD} is provided by Guzman *et al.*, *supra*. Regulation of cell growth by arabinose, in a strain containing a P_{BAD}-*murA* fusion, is demonstrated in Example 2, *infra*.

Regulation is accomplished by fusion of a target gene to a heterologous
15 regulatory element whose expression can be exogenously controlled, for example, by environmental conditions such as chemicals, nutrients, temperature, pH, osmolarity, *etc.* In a preferred embodiment, regulation is such that the level of the target gene product is proportional to the concentration or level of the environmental agent that is used for regulation. In a still more preferred
20 embodiment of the invention, a target gene is fused to P_{BAD} and regulation is achieved by adjusting the concentration of L-arabinose in the growth medium. Low levels of expression are correlated with low concentrations of arabinose and/or the presence of glucose in the medium.

In additional embodiments, regulation is achieved by varying the
25 concentration of an inducer other than arabinose. For example, regulation by maltose is achieved, in cells expressing MalR function, when a target gene is fused to *mal* P_m or *mal* P_x. To provide yet another example, fusion of a target gene to the *raf* regulatory element P_{AGA} allows regulation by raffinose in a cell expressing RafR function. Additional regulatory elements in the *raf* regulatory
30 system include the promoter of the *rafR* gene, P_{rafR}, and the promoter of the *rafE* gene, P_{rafE}. On the basis of these examples, it will be clear to one of skill in the

art that any regulatable promoter, whether positively or negatively regulated, can be used to control the expression of a target gene in response to a substance or environmental condition that regulates that particular promoter.

5 In diploid organisms, controlled regulation of gene expression may not be easy to achieve with a single chromosomal insertion, as it is in prokaryotes. However, in certain situations, mutation or "knockout" of one of the two copies of a target gene may lower expression of the target sufficiently for newly-acquired degrees of drug sensitivity to be obtained. Alternatively, mutation or "knockout" of one copy of a target gene, coupled with controlled expression of the remaining
10 wild-type copy, may be used to achieve heightened drug sensitivity in a diploid. Similarly, situations may be encountered, in both prokaryotes and eukaryotes, in which multiple copies of a gene are present (*e.g.*, ribosomal genes in *E. coli*). In these situations, knockout and/or inactivation of all but one copy of the gene will allow regulation of that remaining functional copy according to the methods of the
15 invention.

Exemplary applications

The methods and compositions of the present invention allow one to control the susceptibility of a cell to a test compound by controlling the amount of
20 a gene product (the target) that is expressed in the cell. This is achieved by adjusting the concentration of an inducer, which will, in turn, regulate the expression of a coding sequence that is fused to a heterologous regulatory element. Sensitivity to a test compound is then determined at various levels of expression of the coding sequence and at different concentrations of the test
25 compound. Expression of lower-than-normal levels of the target will cause a cell to become hyper-susceptible to a compound which interacts with that particular target. Alternatively, a cell expressing lower-than-normal levels of a particular gene product may become susceptible to a compound to which it is not normally susceptible (*i.e.*, to which the cell is not susceptible when it is expressing normal
30 levels of the target). Such a compound is a candidate therapeutic which, following chemical modification, may become capable of inhibiting the viability

of cells expressing normal levels of the target. Techniques for chemical modification of potential therapeutic compounds are well-known to those of skill in the art. *See*, for example, Morin *et al.*, Chemistry and Biology of beta-Lactam Antibiotics, Academic Press, New York, 1982.

5 Many compounds that interact with a target are not active against wild-type cells because an intracellular concentration of the compound cannot be achieved that is sufficient for inhibition, given the concentration of target in the cell. Many factors are responsible for this type of natural resistance, for example, compounds can be hydrolyzed, effluxed, absent because lack of suitable transport,
10 *etc.* *See*, for example, Davies (1994) *Science* 264:375-382; and Nikaido (1994) *Science* 264:382-388. However, inactivation of genes that are involved in natural resistance, for example efflux pumps, allows the construction of mutants that are susceptible to compounds to which wild-type cells are resistant. Such mutants
15 can become hypersusceptible to many unrelated compounds and have been used to characterize novel antimicrobial agents. The methods and compositions of the invention can be used in concert with mutants which display increased susceptibility to compounds because of a mutation in a gene involved in metabolism, transport, efflux, and the like, to identify inhibitors that would not otherwise be detectable. *See* Example 11.

20 Figure 4 shows idealized results for a situation in which a compound inhibits cell viability by interacting with a single monomeric target, and target expression level is regulated. The figure depicts the relationship between inducer concentration and the minimum inhibitory concentration (MIC) of a test compound. The MIC is determined by assessing the minimal concentration of test
25 compound that will inhibit growth, in the presence of a specified concentration of inducer (if applicable), typically using serial two-fold dilutions of test compound. Growth can be recorded, for example, by spectrophotometry or visual inspection of cultures. The minimum amount of test compound that completely inhibits growth, or supports less than 10% growth compared to a control culture, is
30 defined as the MIC. For a wild-type cell, the MIC is constant at all concentrations of inducing agent, because the expression level of the target is not expected to

vary with inducer concentration. For a cell in which target expression is regulated by inducer concentration, there will be a range of inducer concentrations at which MIC is directly proportional to inducer concentration. Thus, lower inducer concentrations, which result in expression of target at levels lower than wild-type, will be correlated with lower MICs than those observed with wild-type cells.

Since this assay relies solely on control of target levels, it provides a screen for candidate therapeutics regardless of whether the function of the target is known. Example 3, *infra*, shows that, for cells in which MurA expression is regulated by arabinose, MIC values both below and above the MIC for wild-type cells can be obtained, when cells are challenged with fosfomycin, a MurA inhibitor.

In certain situations, a target can be part of a multimeric structure composed of different subunits (*e.g.*, a heteromultimer), and a test compound can interact with a sub-region of the multimer contributed by more than one of the subunits, one of which is the target. If target levels are regulated, there will be, in the situation described in the previous paragraph, a certain range of inducer concentrations at which MIC is proportional to inducer concentration. At the target levels specified by this range of inducer concentrations, the target is the limiting component of the multimeric structure. However, with increasing inducer concentration, and concurrent higher target levels, a point will be reached at which the target is no longer the rate-limiting component of the multimer. At this point, the relationship between MIC and inducer concentration reaches a plateau value, which is independent of the MIC for the wild-type strain. This situation is shown schematically in Figure 5.

Prior art screening methods are not applicable to situations in which a particular compound has multiple targets within a cell, with each target having a different degree of sensitivity to the compound. In these cases, methods of the prior art would detect effects only on the target that is most easily inhibited under the assay conditions. The methods of the present invention can be used to control the expression of a target of an inhibitor. If the target is the only cellular gene product that is targeted by the inhibitor, increasing levels of expression of the target will result in higher MICs for the inhibitor (see Figure 4). If the inhibitor

has additional targets, increase of MIC as a function of inducer concentration (*i.e.*, target levels) will reach a plateau value, indicating inhibition of a second target by the inhibitor. Figure 5 shows an idealized depiction of the data that would be obtained in such a situation. Fixing expression of the first (most sensitive) gene product, while varying expression of the other gene product(s), will allow detection of additional targets.

Thus, if a test compound interacts with a single target, the relationship between MIC and inducer concentration will be proportional at all inducer concentrations that are consistent with cell growth. See Examples 3, 4 and 8-13, *infra*. By contrast, if a test compound interacts with multiple targets, or with a structure formed by multiple molecules, one of which is the target, the relationship between MIC and inducer concentration will reach a plateau value at inducer concentrations at and above which the target is no longer the limiting component. Targets can be polypeptides and/or nucleic acids. For example, ribosomes contain both types of target.

Cells can be exposed to any compound that is known in the art or to be synthesized, and the route of exposure can be, for example, by inclusion of the compound in a liquid cell culture medium, by incorporation of the compound into a solid culture medium, or by application of the compound to a solid culture medium, for example, by application to the medium of a porous disc that is saturated with the compound, or by simply pipetting droplets of the compound onto a solid medium.

Use of the invention will allow the rapid identification of potential new therapeutics, such as antibacterial agents. See Example 5, *infra*. Candidates identified by this method can be subjected to chemical modification as known in the art (*see*, for example, Bristol, J.A. (ed.) Annual Reports in Medicinal Chemistry, Academic Press, San Diego) and tested against cells expressing normal levels of the target. Modified compounds that exhibit activity against cells expressing normal target levels are candidate therapeutics.

EXAMPLES

The following examples are intended to illustrate, not to limit the invention.

5 **Example 1: Construction of a strain containing a fusion of P_{BAD} to *murA***

The *murA* gene was selected for testing because it encodes a cytoplasmic protein that is the target of the drug fosfomycin. An *E. coli* strain carrying a single functional copy of the *murA* gene under arabinose control in the
10 chromosome was constructed. In this strain, the level of expression of *murA* is controlled by the amount of arabinose present in the medium. In addition, this strain is dependent on arabinose because this sugar is required to induce the expression of the essential gene *murA*, and this strain cannot metabolize arabinose because the catabolic genes have been deleted ($\Delta(araCBA)araD$). The
15 susceptibility of the strain to fosfomycin, a uridine diphospho-N-acetyl-D-glucosamine enolpyruvyl transferase (MurA) inhibitor, was tested at different concentrations of arabinose. Susceptibility to unrelated antibiotics that inhibit other targets, *i.e.*, tetracycline (a protein synthesis inhibitor) and ciprofloxacin (a DNA gyrase inhibitor), was also investigated. A new inhibitor of MurA was
20 identified by the practice of the invention. See Example 5.

The *E. coli* strains used for this example are *E. coli* VECO2042 (*pir*⁺, *recA*), *E. coli* VECO2054 ($\Delta(araCBA)araD$) and *E. coli* VECO2055 ($\Delta(araCBA)araD$) *PmurA::Km-araC-P_{BAD}*).

VECO2055 was constructed as follows:

25 Allele replacement requires a double recombination event to occur. Two regions of homology used for recombination were the *murA* coding region and 400 base pairs of DNA immediately upstream of *murA*. The chromosomal replacement cassette and the strategy used to replace wild type *murA* with P_{BAD}-*murA* is diagrammed in Figure 6.

30 A DNA sequence containing *murA* was PCR-amplified from *E. coli* strain JM109 chromosomal DNA using oligonucleotides DYV-055 (SEQ ID NO. 3,

Table 2) and DYV-056 (SEQ ID NO. 4, Table 2), and cloned as an NcoI/XbaI fragment into the expression vector pBAD-MycHisB (Invitrogen Corporation, Carlsbad, CA).

400 base pairs of upstream *murA* sequence was PCR amplified from *E. coli* strain JM109 chromosomal DNA using oligonucleotides DYV-057 (SEQ ID NO. 5, Table 2) and DYV-058 (SEQ ID NO. 6, Table 2), and directly cloned into pCR2.1 (Invitrogen Corporation, Carlsbad, CA).

The suicide vector pWM95 (Metcalf *et al.* (1996) *Plasmid* 35:1-13) was chosen to perform the allele replacement procedure. pWM95 is an ampicillin-resistant, conditionally replicative plasmid requiring the *pir* gene in *trans* for plasmid replication to occur. pWM95 also carries the *sacB* gene which confers sucrose sensitivity to transformed strains grown in the presence of sucrose. When this plasmid is introduced into a host that does not supply the Pir protein, strains carrying chromosomal integrants can be selected. The *sacB* gene then allows for selecting plasmid-free segregants as sucrose-resistant clones. *E. coli* strain VECO2042 (*pir*⁺, *recA*) was used for all cloning steps with the conditionally replicative pWM95 and its derivatives.

The *araC*-P_{BAD}-*murA* and upstream *mur* sequences were cloned into the suicide vector pWM95 by three-way ligation to create pDY-10. The kanamycin resistance gene from plasmid pBSL99 (ATCC 87141) was cloned as a HindIII fragment into pDY10 to create pDY11.

pDY11 was introduced into *E. coli* strain VECO2054. Transformants were plated on LB plates supplemented with kanamycin (25 µg/ml) and ampicillin (100 µg/ml) and incubated at 37°C overnight. A number of transformants were streaked onto LB plates supplemented with kanamycin (25 µg/ml) and arabinose (0.2%) and incubated at 37°C overnight. Isolated colonies were then streaked onto LB plates supplemented with kanamycin (25 µg/ml), sucrose (6%), and arabinose (0.2%) to select for sucrose-resistant recombinants. NaCl was omitted from LB plates during sucrose resistance selection. Sucrose-resistant recombinants were screened for ampicillin sensitivity and arabinose growth dependence. Chromosomal replacement in candidate clones was verified by

checking the chromosomal junctions with PCR primer pairs DYV-070 / DYV-073 (SEQ ID NO: 7/SEQ ID NO: 9) and DYV-082/DYV-071 (SEQ ID NO: 10/SEQ ID NO: 8).

5

Table 2. Oligonucleotides used for PCR

Primer	Sequence (5' -> 3')	SEQ ID NO.
DYV-055	GGCCATGGATAAATTTTCGTGTTTCAGG	3
DYV-056	GGTCTAGATTATTTCGCCTTTCACACGC	4
DYV-057	GGACCCGGGTCTGATTTATCAGCGAGGC	5
DYV-058	GCCATATGTCCGGAAGCTTAGTTTGTTCCTCAGTTAAC	6
DYV-070	CCGGATATGGCGTTAACCG	7
DYV-071	CCCATGGTTCCAGTAAGTTCC	8
DYV-073	GTGAATGATGTAGCCGTC	9
DYV-082	CTCGCTAACCAAACCGGTAACC	10

Note: Underlined sequences correspond to non-complementary bases.

Example 2: Regulation of growth of VECO2055 by arabinose

10 Growth of the $P_{BAD-murA}$ fusion strain (*E. coli* VECO2055) was tested as a function of arabinose concentration. Figure 7 shows that growth of the fusion strain is dependent on arabinose concentration, demonstrating the regulation of *murA* by a heterologous regulatory element and indicating that, at low arabinose concentrations, *murA* function is limiting for cell growth.

15

Example 3: Susceptibility of VECO2055 to fosfomycin

An experiment was conducted, using the $P_{BAD-murA}$ fusion strain (VECO2055) and its parent strain (VECO2054), to compare their susceptibility to fosfomycin at different arabinose concentrations. Fosfomycin is an antibiotic which targets the *murA* gene product. Kahan *et al.* (1974) *Ann. NY Acad. Sci.* 235:364-386.

20

Preparation of inoculum

Cells were grown overnight in 5 ml of LB supplemented with 0.1% arabinose on a rotary shaker at 35°C and 200 rpm. 100 µl of overnight culture was collected, centrifuged for 5 min. at room temperature at 14,000 rpm, and the

cell pellet was suspended in 1 ml of LB with no added arabinose. The cell suspension was diluted 1:1000 in LB and used as inoculum.

Preparation of 96 well plates for checkerboard assay

Checkerboard assays were performed in 96-well microtiter plates, in which arabinose concentration was varied in the first dimension and fosfomycin concentration was varied in the second dimension. Fosfomycin concentrations varied by two-fold between rows; dilutions were performed in LB supplemented with different concentrations of arabinose, or lacking arabinose. A control row lacking fosfomycin, and a control column lacking arabinose, were also included.

Total volume in each well was 50 μ l.

Inoculation of plates and incubation

50 μ l of inoculum, (*i.e.*, diluted cell suspension) was added to each well. After 20 hours of incubation at 35°C, cell growth was measured in each well and compared to wells with no inoculum.

The results, presented in Figure 8, show the minimum inhibitory concentration (MIC) of fosfomycin, as a function of arabinose concentration, for the P_{BAD} -*murA* fusion strain *E. coli* VECO2055, compared to wild-type. The results demonstrate that MIC values above and below the MIC for wild-type cells can be attained in the fusion strain through adjustment of arabinose levels.

20

Example 4: Comparison of susceptibility of VECO2055 to fosfomycin with susceptibility to antibiotics which do not target the *murA* gene product

An experiment was conducted on the P_{BAD} -*murA* fusion strain (*E. coli* VECO2055) to compare its susceptibility to fosfomycin with its susceptibility to several other antibiotics (tetracycline and ciprofloxacin) which do not target the *murA* gene product.

25

Preparation of inoculum

Cell culture and preparation of inocula were performed as described in Example 3, *supra*.

Preparation of 96 well plates for checkerboard assay

Checkerboard assays were performed in 96-well microtiter plates, in which arabinose concentration was varied in the first dimension and antibiotic concentration was varied in the second dimension. Antibiotic concentrations varied by two-fold between rows; dilutions were performed in LB supplemented with different concentrations of arabinose or lacking arabinose. A control row lacking antibiotic, and a control column lacking arabinose, were also included. Total volume in each well was 50 μ l. Similar assays were conducted, using fosfomycin, tetracycline or ciprofloxacin, to test the influence of arabinose on susceptibility to these antibiotics.

Inoculation of plates and incubation

50 μ l of inoculum, (*i.e.*, diluted cell suspension) was added to each well. After 20 hours of incubation at 35°C, cell growth was measured in each well and compared to wells with no inoculum. Figure 9 shows that the susceptibility of strain VECO2055 to tetracycline and ciprofloxacin was independent of the presence of arabinose in the medium. The variation observed for sensitivity to ciprofloxacin and tetracycline is typical of that obtained in a MIC determination. Sensitivity to fosfomycin was dependent on arabinose concentration, confirming the results shown in Figure 8. The minimum concentration of arabinose needed to support growth was $5 \times 10^{-5}\%$.

The susceptibility of strain VECO2055 to fosfomycin was strongly associated with the amount of arabinose present in the medium. MICs comparable to the wild type were achievable when sufficient arabinose was added to the medium. Cells became more susceptible to fosfomycin at lower concentrations, in a concentration-dependent manner. The difference in susceptibility between the lowest and highest MICs was approximately 100-fold. The susceptibility to tetracycline and ciprofloxacin remained constant and was independent of arabinose concentration.

Example 5: Identification and validation of a new inhibitor of MurA**Preparation of inoculum**

Cell culture and preparation of inocula were performed as described in Example 3, *supra*. *E. coli* VECO2055 was used in all experiments.

5 Screening

Microtiter plates were used for screening. Wells contained LB with 0, 0.0004, or 0.002% arabinose. Eighty unrelated compounds were tested; test compounds were added to the wells at final concentrations of 2, 4 and 8 µg/ml. 50 µl of inoculum was added to each well and the plates were incubated at 35°C.

10 Growth was measured after 24 and 40 hours. The growth of cells incubated at the higher arabinose concentration (0.002%) was not inhibited by any of the test compounds. When cells were incubated at the lower arabinose concentration (0.0004%), compound 47-7-70 was the only test compound that inhibited growth at all three concentrations tested. As a control, the MIC for fosfomycin was
15 determined at each arabinose concentration.

Inhibition of MurA activity

The enzymatic activity of purified MurA was assayed in the presence of the same test compounds used in the screen described *supra*. Different concentrations of the test compounds were added to buffer (50 mM Tris-HCl, pH
20 8; 0.2 mM UDP-N-acetylglucosamine) containing 7 µg/ml MurA. The reaction was started by addition of phosphoenolpyruvate to 0.1 mM, and the reaction mixture was incubated for 30 min at 25°C. Released phosphate was measured with malachite green reagent, and quantitated by spectrophotometry. Only
25 compound 47-7-70 showed inhibitory activity, with an IC₅₀ of 8 µg/ml.

25 Susceptibility of VECO2055 to compound 47-7-70 as a function of arabinose concentration

A checkerboard assay was performed in a 96-well microtiter plate, similar to that described in Example 3, *supra*. Concentration of compound 47-7-70 was varied in one dimension, and arabinose concentration was varied in the other
30 dimension. A control row lacking compound 47-7-70, and a control column

lacking arabinose, were also included on the plate. In addition, a control plate containing dilutions of fosfomycin instead of compound 47-7-70 was also tested.

50 μ l of VECO2055 inoculum, prepared as described in Example 3, *supra*, was added to each well. The plates were incubated 20 hours at 35°C, at which time cell growth was measured and compared to that in wells that had not received an inoculum. The results are presented in Table 3. As can be seen, increasing susceptibility of VECO2055 to compound 47-7-70 was correlated with lower concentrations of arabinose in the medium, as expected for a compound that blocks cell growth by inhibition of the MurA enzyme.

Table 3: MIC of compound 47-7-70 as a function of arabinose concentration for VECO2055

% arabinose in medium	MIC of 47-7-70 (μ g/ml)
0.00018	2
0.000375	4
0.00075	8
0.0015	16
0.00312	16

Example 6: Construction and properties of a tightly regulated maltose regulatory system

MalR is a repressor that controls the expression of the maltosaccharide regulon in *S. pneumoniae* and belongs to the LacI-GalR family of repressors. Two operons are regulated in opposite direction, *malXCD* (*Px* promoter) and *malMP* (*Pm* promoter), see Figure 1 (SEQ ID NO: 1). Affinity of the MalR for *Pm* is higher than for *Px* and, in both cases, a high basal level of expression has been reported. Nieto *et al.* (1997) *J. Biol. Chem.* 272:30860-30865. This example shows that tighter regulation of the *Px* promoter can be obtained by modifying the repressor site and by growing cells in minimal medium.

The *S. pneumoniae* strains used for this example are *S. pneumoniae* VSPN3026, *S. pneumoniae* VSPN3021 with *katA* under *Px* control, *S.*

pneumoniae VSPN3025 with *katA* under modified *Px* control, and *S. pneumoniae* VSPN3022 with *katA* under *Px* control, with *Pm* upstream.

Construction of VSPN3021

Px was PCR amplified from VSPN3026 using oligonucleotides MAL1 and
5 MAL2 (Table 4), and cloned into the T-tailed PinPoint Xa-1 T-Vector (Promega Corporation, Madison, WI). The construct was digested with EcoRI and BamHI, and the 387 bp *Px*-containing fragment was cloned into pR326 vector to create pR326MX. The reporter gene used for measuring expression was the gene for catalase, *katA*, from *B. subtilis* ATCC 6633. The gene encoding *katA* was PCR-
10 amplified using oligonucleotides KAT1 and KAT2 (Table 4) and cloned into the NdeI site of pR326MX to create pR326MXK.

An additional DNA sequence was added to the construct to target the insertion into a non-essential DNA sequence of the *S. pneumoniae* chromosome. For this purpose, a 300 bp fragment of the *cpbA* gene was PCR-amplified from
15 the DNA of *S. pneumoniae* VSPN3026, using oligonucleotides CPB1 and CPB2 (Table 4), and the amplification product was inserted into the ClaI site of pR326MXK to create pR326MXKC. This plasmid was used to transform VSPN3026 and construct a *S. pneumoniae* strain, VSPN3021, that carries the insertion in the chromosome, according to the insertional duplication mutagenesis
20 method of Claverys *et al.* (1995) *Gene* 164:123-128.

Construction of VSPN3025

A DNA sequence containing the repressor binding site of *Px* was mutagenized, to convert a GGA to a GCG (see Figure 1), by using the QuickChange site directed mutagenesis kit (Stratagene, La Jolla, CA). The
25 oligonucleotides MAL6 and MAL6C (Table 4) were used as primers, and pR326MXKC was used as template. Note that MAL6C includes the mutant sequence. The resultant plasmid, with a mutation in *Px*, was called pR326MMXKC. This plasmid was used to transform VSPN3026 and construct a *S. pneumoniae* strain, VSPN3025, that carries the insertion in the chromosome.

Construction of VSPN3022

The *Px* and *Pm* regulatory region was PCR amplified from VSPN3026 using oligonucleotides MAL3 and MAL2 (Table 4), and cloned into the T-tailed PinPoint Xa-1 T-Vector (Promega Corporation, Madison, WI). The construct was digested with EcoRI and BamHI, and the *Px* and *Pm*-containing fragment was cloned into the pR326 vector to create pR326MXM. The catalase gene was inserted under the control of this regulatory region as described above to create pR326MXMKC. This plasmid was used to transform VSPN3026 and construct a *S. pneumoniae* strain, VSPN3022, that carries the insertion in the chromosome.

Table 4. Oligonucleotides used in *mal* constructions

Oligo	Sequence (5' -> 3')	SEQ ID NO
MAL1	TAGGTTGAATTCATAGAAAATAGATAGGGATTAGAACCA GGG	11
MAL2	TGCGAGGATCCTACTTGTCGTCGTCGTCCTTGTAGTCGAT ATCATATGTATTCCTCCCAAAGAATAGCAAGT	12
KAT1	CCATCGCATATGAGTTCAAATAAACTGACAAC	13
KAT2	CACGACATATGAATCTTTTTTAATCGGCAATCC	14
CBP1	CTGAATCGATGCAGCCACTTCTTCTAATATGGC	15
CBP2	AGCTATCGATTTTCTAACCTTGTAGCCTCAGC	16
MAL3	TAGGTTGAATTCTCGTGTGTTAAATAATG	17
MAL6	CGCAAACGTTTGCGTTTATGAGCTTAG	18
MAL6C	CTAAGCTCATAAACGCAAACGTTTGCG	19

Catalase assay

Catalase activity was measured in cell cultures grown in medium C+Y without glucose (Tomasz (1970) *J Bacteriol.* 101:860-871.) and minimal medium CDEN without glucose. Rane *et al.* (1940) *J. Bacteriol.* 40:695-704. Cells were collected in mid-logarithmic phase, centrifuged, and resuspended in 20 mM Tris-HCl buffer, pH 8, containing 0.25% Triton-X100. After autolysis of cells, 10 μ l of extract were added to 1 ml of 1.5 mM H₂O₂, and the reaction was followed fluorometrically with scopoletin. One unit of catalase activity is 1 μ mol H₂O₂ hydrolysis per min. at 22°C (see Table 5).

Table 5. Catalase activity in *mal* insertion strains

Strain	Growth conditions			
	C+Y (2% maltose)	C+Y (No maltose)	CDEN (2% maltose)	CDEN (No maltose)
VSPN3022	43	14	69	1.12
VSPN3021	60	16	62	13
VSPN3025	65	6	66	0.35

5

The data (expressed as units of catalase activity) show that tighter regulation of the maltose regulatory system can be obtained when using minimal medium (CDEN), and that the tightest regulation is obtained when using the modified *P_x* promoter (VSPN3025).

10

Example 7: Raffinose-regulated expression of the *S. pneumoniae spi* gene by the *S. pneumoniae aga* promoter

A. Construction of transcriptional fusions

A DNA sequence containing *S. pneumoniae rafR* and *P_{AGA}* (*aga* promoter) was PCR-amplified from *S. pneumoniae* VSPN3026 chromosomal DNA using oligonucleotides REGAGAEI5' (SEQ ID NO. 20, Table 6) and REGAGANB3' (SEQ ID NO. 21, Table 6) and cloned as an EcoRI/NdeI fragment into the integration vector pR326 (Claverys, *et al.*, *supra*) to generate plasmid pR326RafRPaga. A DNA fragment containing the first 270 bp of the leader peptidase gene (*spi*) from *S. pneumoniae* R6 chromosomal DNA was PCR-amplified using oligonucleotides MALSPI5' (SEQ ID NO. 22, Table 6) and MALSPI3' (SEQ ID NO. 23, Table 6) and cloned as an NdeI/BamHI fragment into plasmid pR326RafRPaga, resulting in plasmid pR326RPASPI.

Using this plasmid as a template, a DNA fragment containing only *P_{AGA}* and *spi* sequences was amplified using oligonucleotides Paga5' EI (SEQ ID NO. 24, Table 6) and MALSPI3' (SEQ ID NO. 23, Table 6) and cloned into the T-tailed pGEM-T Easy Vector (Promega Corporation, Madison, WI). The construct was digested with EcoRI and the *P_{AGA}-spi* containing fragment was cloned into

the integration vector pR326 to create PR326PagaSpi. This plasmid was used to transform VSPN3026, that had been grown in C+Y lacking sucrose and supplemented with 0.2% raffinose (C+Y+Raf). Transformants were plated on TSA-sheep blood plates supplemented with chloramphenicol (2.5 µg/ml) and raffinose (0.2%) and incubated at 37°C/5% CO₂ overnight. Chloramphenicol-resistant strains were cultured in C+Y+Raf medium.

The site of insertion was verified in one of the isolates, *S. pneumoniae* strain VSPN3041. Insertion in the targeted site in the *spi* gene was verified by PCR using the primers Paga100 (SEQ ID NO. 25, Table 6) and Spi3'SPn (SEQ ID NO. 26, Table 6). This analysis indicated that VSPN 3041 carried a truncated 270 bp *spi* gene under natural promoter control and a complete *spi* gene under *P_{AGA}* control.

Table 6: Sequences of oligonucleotides used for construction of a *P_{AGA}-spi* fusion and targeting of the fusion to the chromosomal *spi* gene

Oligonucleotide	Sequence (5' → 3')	SEQ ID NO.
REGAGAEI5'	CCCGGAATTCAGCTTGGTAGGATTCATAA TGTTGCC	20
REGAGANB3'	GCCGCGGATCCGCGCATATGCATTTACTTC ACCTCATCACTTTATTG	21
MALSPI5'	GGGGAATTCCATATGAATTTATTTAAAAAT TTCTTAAAAGAGTGGG	22
MALSPI3'	GCGCTCTAGATCATTTTCGTAACGAATGGT GTCG	23
Paga5'EI	GCGCCGGAATTCCATGTGCTACCTCCTACCT AACATTTTACC	24
Paga100	CTCCTACCTAACATTTTACCAT	25
Spi3'Spn	TTAAAATGTTCCGATACGGGTGATTGG	26

B. Regulation of growth of VSPN3041 by raffinose

Since VSPN3041 carries an essential gene (*spi*) under the control of *P_{AGA}*, the strain should be dependent on raffinose for growth. Accordingly, the effect of

raffinose on the growth of VSPN3041 and the parent isogenic strain VSPN3026 was compared.

5 Preparation of 96 well plates: 96-well microtiter plates were used for the experiments. Serial twofold dilutions of raffinose (in C+Y medium) were performed across columns, including one column in which no raffinose was added. Total volume in each well was 50 μ l.

10 Preparation of inoculum: VSPN3041 and VSPN3026 were grown for 4 hours at 37° C in 5 ml C+Y+raf. Ten microliters of the culture was added to 10 ml C+Y containing 1% sucrose, incubated for 6 hours at 37° C, then frozen as a 15% glycerol solution. A 1:10 dilution of the frozen stock in C+Y lacking sucrose, containing 2×10^6 colony forming units/ml, was used as inoculum.

Growth of bacteria in 96-well plates: 50 μ l of inoculum was added to each well, to give a final volume of 100 μ l. Plates were incubated at 35° C and growth was monitored every hour up to 10 hours.

15 Results: Figure 10 shows the growth of VSPN3041 at different raffinose concentrations, measured by optical density. It is clear that growth of the fusion strain is dependent on raffinose concentration, demonstrating that an essential gene is regulated by raffinose in VSPN3041. In the experiment shown in Figure 11, the growth of VSPN3041 on raffinose is compared to growth on sucrose (each sugar present in medium at 0.2% w/v). The results of this experiment indicate
20 that VSPN3041 does not grow in the absence of raffinose, again demonstrating that an essential gene is positively regulated by raffinose in this strain. Figure 12 compares the growth of VSPN3041 and the parent isogenic strain VSPN3026, at different raffinose concentrations. The results indicate that VSPN3041 is
25 raffinose dependent, while the growth of the parent strain is not dependent on raffinose.

 The raffinose-dependent phenotype of VSPN3041, compared to its parent strain, indicates that *P_{AGA}* controls the expression of an essential gene in VSPN3041. Given that the difference between VSPN3041 and its parent is an
30 insertion that places the *spi* gene under *P_{AGA}* control, the essential raffinose-regulated gene in VSPN3041 is the *spi* gene (or a gene downstream of *spi*).

Hence, the *spi* gene (or its downstream gene) in VSPN3041, regulated by the P_{AGA} promoter in response to raffinose, is limiting for cell growth at low raffinose concentrations. Since growth is dependent on the expression of this essential gene and the level of induction can be controlled, the growth of VSPN3041 can be
5 controlled by the induction or repression of the P_{AGA} promoter.

Example 8: Construction and properties of a strain containing a P_{BAD} -*def* transcriptional fusion

A Construction of P_{BAD} -*def* transcriptional fusions in *E. coli*

10 The product of the *def* gene, the enzyme peptidyl deformylase, plays a major role in protein synthesis in bacteria. A DNA sequence containing the full-length *def* gene was PCR-amplified from chromosomal DNA of *E. coli* strain JM109 using oligonucleotides DYV-157 (SEQ ID NO: 27) and DYV-158 (SEQ ID NO: 28), and cloned as a NcoI/BglII fragment into expression vector pBAD-
15 MycHisB (Invitrogen Corporation, Carlsbad, CA), to create pDY8.
Oligonucleotide sequences are given in Table 7.

pDY20 was created by PCR-amplification of the kanamycin resistance cassette from plasmid pBSL99 with the primers DYV-087 (SEQ ID NO: 35) and DYV-088 (SEQ ID NO: 36) and cloned into pBlueScriptSKII⁺ (Stratagene, La
20 Jolla, CA) as an XbaI/SacI fragment. 600 base pairs of upstream *def* sequence were PCR amplified from *E. coli* strain JM109 chromosomal DNA using oligonucleotides DYV-155 (SEQ ID NO: 29) and DYV-156 (SEQ ID NO: 30), and cloned as a SacI/AscI fragment into vector pDY20 to create pDY9.
Oligonucleotide sequences are given in Table 7.

25 The suicide vector pKO3 (Link *et al.*, *supra*) was chosen to perform the allele replacement procedure with the *def* gene. pKO3 is a chloramphenicol-resistant vector containing the temperature-sensitive pSC101 origin of replication and the *sacB* gene for counter-selection. pKO3-derived plasmids are incapable of autonomous replication at 43°C. When a host strain harboring a pKO3 construct
30 is plated at 43°C on media containing chloramphenicol, chromosomal integrants can be selected. Integration of a pKO3 construct into a host chromosome at

elevated temperature occurs via homologous recombination between *E. coli* DNA cloned into pKO3 and the *E. coli* chromosome.

The *araC*-P_{BAD}-*def* cassette was excised as an NdeI/BglII fragment from pDY8, and the upstream *def*-Kanamycin cassette was excised as an Ecl136 II/NdeI fragment from pDY9. The purified fragments were cloned in a three-way ligation with SmaI/BamHI digested pKO3 to create pDY15.

pDY15 was introduced into *E. coli* strain VECO2054. Transformants were selected on LB plates supplemented with chloramphenicol (25 µg/ml) and kanamycin (25 µg/ml), incubated at 30°C overnight. A number of transformants were streaked onto LB plates supplemented with chloramphenicol (25 µg/ml), kanamycin (25 µg/ml) and arabinose (0.2%) and incubated at 43°C overnight. Isolated colonies were then streaked onto LB plates supplemented with kanamycin (25 µg/ml) and arabinose (0.2%) and incubated at 37°C overnight. Isolated colonies were then streaked onto the same medium (LB+kan+ara) supplemented with 6% sucrose and incubated at 37°C overnight to select for sucrose-resistant recombinants. NaCl was omitted from LB plates during this sucrose resistance selection step. Sucrose-resistant recombinants were screened for chloramphenicol sensitivity and arabinose-dependent growth. Chromosomal replacement of the *def* gene in a clone, VECO2065 (*araC*-P_{BAD}-*def*), was verified by assaying for specific PCR products, derived from the chromosomal junctions, with PCR primer pairs DYV-069 (SEQ ID NO: 37)/DYV-082 (SEQ ID NO: 10) and DYV-073 (SEQ ID NO: 9)/DYV-155 (SEQ ID NO: 29). See Tables 2 and 7 for primer sequences.

B. Susceptibility of VECO2065 (P_{BAD}-*def*) strain to VRC483 and other antimicrobial agents

An experiment was conducted using the VECO2065 strain and its parent strain (VECO2054), to compare their susceptibility to VRC483 over a range of arabinose concentrations. VRC483 is a compound with antibacterial activity that targets the *def* gene product. This compound was identified in a deformylase screen at Versicor and the IC₅₀ is 11 nM for *E. coli* deformylase. Deformylase activity was measured as described. Rajagopalan *et al* (1997) *Biochemistry*

36:13910-13918. The susceptibility of VECO2065 to the unrelated antibiotics fosfomycin and ciprofloxacin was also tested.

1. Preparation of inoculum. Cells were grown overnight in 5 ml of LB supplemented with 0.1 % arabinose, on a rotary shaker at 35°C and 200 rpm.

5 100 µl of overnight culture was collected, centrifuged at 14,000 rpm for 5 min at room temperature, and the cell pellet was suspended in 1 ml of medium with no added arabinose. The cell suspension was diluted 1:1000 in medium and used as inoculum.

2. Preparation of 96 well plates. Checkerboard assays were performed in
10 96-well microtiter plates, in which arabinose concentration was varied in the first dimension, and the concentration of antimicrobial compound in the second. The concentration of antimicrobial varied by two-fold between rows. Dilutions were performed in medium supplemented with different concentrations of arabinose, or medium lacking arabinose. A control row lacking antimicrobial, and a control
15 column lacking arabinose, were also included. Total volume in each well was 50 µl.

3. Inoculation of plates and incubation. 50 µl of inoculum (*i.e.*, diluted cell suspension) was added to each well. After 20 hours of incubation at 35°C, cell growth was measured in each well and compared to wells with no inoculum.

20 4. Results. Figure 13 shows the minimum inhibitory concentration (MIC) of VRC483, as a function of arabinose concentration, for the $P_{BAD-def}$ strain VECO2065. The parent wild-type strain (VECO2054) was not susceptible to VRC483 in the range tested. VECO2065 was susceptible to VRC483 at low arabinose concentrations, and the susceptibility was inversely related to the
25 inducer concentration. Susceptibility of VECO2065 to compounds that do not target the product of the *def* gene, such as fosfomycin and ciprofloxacin, did not change with arabinose concentration.

Example 9: Construction and properties of a strain containing a P_{BAD}-*folA* transcriptional fusion

A Construction of P_{BAD}-*folA* transcriptional fusions in *E. coli*

The product of the *folA* gene is a dihydrofolate reductase. This enzyme is involved in folate synthesis in bacteria. A DNA sequence containing the full-length *folA* gene was PCR-amplified from the chromosomal DNA of *E. coli* strain JM109, using oligonucleotides DYV-095 (SEQ ID NO: 31) and DYV-096 (SEQ ID NO: 32), and cloned as an NcoI/BglII fragment into expression vector pBAD-MycHisB (Invitrogen Corporation, Carlsbad, CA), to create pDY5.

Oligonucleotide sequences are given in Table 7.

Six hundred base pairs of upstream *folA* sequence were PCR amplified from *E. coli* strain JM109 chromosomal DNA, using oligonucleotides DYV-093 (SEQ ID NO: 33) and DYV-094 (SEQ ID NO: 34), and cloned as a SacI/AscI fragment into vector pDY20 (see Example 8) to create pDY6. Oligonucleotide sequences are given in Table 7.

The *araC*-P_{BAD}-*folA* cassette was excised as a NdeI/BglII fragment from pDY5, and the upstream *folA*-kanamycin cassette was excised as an Ecl136 II/NdeI fragment from pDY6. The purified fragments were cloned in a three-way ligation with SmaI/BamHI digested pWM95 to create pDY42.

pDY42 was introduced into *E. coli* strain VECO2054. Transformants were selected on LB plates supplemented with ampicillin (100 µg/ml) and kanamycin (25 µg/ml), incubated at 37°C overnight. A number of transformants were streaked onto LB plates supplemented with kanamycin (25 µg/ml) and arabinose (0.2%) and incubated at 37°C overnight. Isolated colonies were picked and re-streaked onto LB plates supplemented with kanamycin (25 µg/ml) and arabinose (0.2%) and incubated at 37°C overnight. Isolated colonies were then streaked onto the same medium (LB+kan+ara) supplemented with 6% sucrose and incubated at 37°C for 24 hours. The plates were then incubated for an additional 24 hours at room temperature to select for sucrose-resistant recombinants. NaCl was omitted from LB plates during this selection for sucrose resistance. Sucrose-resistant recombinants were screened for ampicillin sensitivity and arabinose-

dependent growth. Chromosomal replacement of the *folA* gene in a clone, VECO2079 (*araC-P_{BAD}-folA*), was verified by assaying for specific PCR products, derived from the chromosomal junctions, with PCR primer pairs DYV-093 (SEQ ID NO: 33)/DYV-163 (SEQ ID NO: 38) and DYV-107 (SEQ ID NO: 39)/DYV-218 (SEQ ID NO: 40). See Table 7 for primer sequences.

B. Susceptibility of VECO2079 (*P_{BAD}-folA*) strain to trimethoprim and other antimicrobial agents

An experiment was conducted using the VECO2079 strain and its parent strain (VECO2054), to compare their susceptibility to trimethoprim over a range of arabinose concentrations. trimethoprim is a compound with antibacterial activity that targets dihydrofolate reductase, the product of the *folA* gene. Huovinen *et al.* (1995) *Antimicrob. Agents Chemother.* **39**(2):279-289. The susceptibility of VECO2079 to the unrelated antibiotics fosfomycin and ciprofloxacin was also tested.

1. Preparation of inoculum. Cells were grown overnight in 5 ml of LB supplemented with 0.1 % arabinose, on a rotary shaker at 35°C and 200 rpm. 100 µl of overnight culture was collected, centrifuged at 14,000 rpm for 5 min at room temperature, and the cell pellet was suspended in 1 ml of medium with no added arabinose. The cell suspension was diluted 1:1000 in medium and used as inoculum.

2. Preparation of 96 well plates. Checkerboard assays were performed in 96-well microtiter plates, in which arabinose concentration was varied in the first dimension, and the concentration of antimicrobial compound in the second. The concentration of antimicrobial varied by two-fold between rows. Dilutions were performed in medium supplemented with different concentrations of arabinose, or medium lacking arabinose. A control row lacking antimicrobial, and a control column lacking arabinose, were also included. Total volume in each well was 50 µl.

3. Inoculation of plates and incubation. 50 µl of inoculum (*i.e.*, diluted cell suspension) was added to each well. After 20 hours of incubation at 35°C, cell growth was measured in each well and compared to wells with no inoculum.

4. Results. Figure 14 shows the minimum inhibitory concentration (MIC) of trimethoprim, as a function of arabinose concentration, for the P_{BAD} -*folA* strain (VECO2079) compared to the parent wild-type strain, VECO2054. The results show that the MIC of trimethoprim, a *folA* inhibitor, was dependent on arabinose concentration in the P_{BAD} -*folA* strain (VECO2079); while MIC values of trimethoprim for the wild-type strain were not dependent on arabinose concentration. Figure 14 also shows that susceptibility of VECO2079 to compounds that do not target the product of the *folA* gene, such as fosfomycin and ciprofloxacin, did not change with arabinose concentration.

Example 10: Construction and properties of a strain containing a P_{BAD} -*gyrB* transcriptional fusion

A. Construction of P_{BAD} -*gyrB* transcriptional fusions in *E. coli*.

The product of the *gyrB* gene is the beta subunit of gyrase, a bacterial DNA topoisomerase. A DNA sequence containing the full-length *gyrB* gene was PCR-amplified from the chromosomal DNA of *E. coli* strain JM109, using oligonucleotides DYV-099 (SEQ ID NO: 41) and DYV-204 (SEQ ID NO: 42), and cloned as an NcoI/PstI fragment into expression vector pBAD-MycHisB (Invitrogen Corporation, Carlsbad, CA) to create pDY34. Oligonucleotide sequences are given in Table 7.

Six hundred base pairs of upstream *gyrB* sequence were PCR-amplified from *E. coli* strain JM109 chromosomal DNA using oligonucleotides DYV-097 (SEQ ID NO: 43) and DYV-098 (SEQ ID NO: 44), and cloned as a SacI/AscI fragment into vector pDY20 (see Example 8) to create pDY38. Oligonucleotide sequences are given in Table 7.

The *araC*- P_{BAD} -*gyrB* cassette was excised as an NdeI/ Xba fragment from pDY34, and the upstream *gyrB*-kanamycin cassette was excised as an Ecl136 II/ NdeI fragment from pDY38. The purified fragments were cloned in a three-way ligation with SmaI/XbaI digested pWM95 to create pDY40.

pDY40 was introduced into *E. coli* strain VECO2054. Transformants were selected on LB plates supplemented with ampicillin (100 µg/ml) and

kanamycin (25 µg/ml), incubated at 37°C overnight. A number of transformants were streaked onto LB plates supplemented with kanamycin (25 µg/ml) and arabinose (0.2%) and incubated at 37°C overnight. Isolated colonies were picked and re-streaked onto the same medium (LB+kan+ara) and incubated at 37°C overnight. Isolated colonies were then streaked onto the same medium (LB+kan+ara) supplemented with 6% sucrose and incubated at 37°C for 24 hours. The plates were then incubated for an additional 24 hours at room temperature to select for sucrose-resistant recombinants. NaCl was omitted from LB plates during this selection for sucrose resistance. Sucrose-resistant recombinants were screened for ampicillin sensitivity and arabinose-dependent growth. Chromosomal replacement of the *gyrB* gene in a clone, VECO2083 (*araC*-P_{BAD}-*gyrB*), was verified by assaying for specific PCR products, derived from the chromosomal junctions, with PCR primer pairs DYV-211 (SEQ ID NO: 45)/DYV-163 (SEQ ID NO: 38) and DYV107 (SEQ ID NO: 39)/DYV-214 (SEQ ID NO: 46). See Table 7 for primer sequences.

B. Susceptibility of VECO2083 (P_{BAD}-*gyrB*) strain to novobiocin and other antimicrobial agents

The susceptibility of VECO2083 strain to novobiocin, and to the unrelated antibiotics fosfomycin and ciprofloxacin, was tested. Novobiocin is an antibiotic of the coumarin group that inhibits gyrase by binding to the *gyrB* gene product. Maxwell (1993) *Mol Microbiol.* 9(4):681-686.

1. Preparation of inoculum. Cells were grown overnight in 5 ml of LB supplemented with 0.1 % arabinose, on a rotary shaker at 35°C and 200 rpm. 100 µl of overnight culture was collected, centrifuged at 14,000 rpm for 5 min at room temperature, and the cell pellet was suspended in 1 ml of medium with no added arabinose. The cell suspension was diluted 1:1000 in medium and used as inoculum.

2. Preparation of 96 well plates. Checkerboard assays were performed in 96-well microtiter plates, in which arabinose concentration was varied in the first dimension, and concentration of antimicrobial compound in the second. The concentration of antimicrobial varied by two-fold between rows. Dilutions were

performed in medium supplemented with different concentrations of arabinose, or medium lacking arabinose. A control row lacking antimicrobial, and a control column lacking arabinose, were also included. Total volume in each well was 50 μ l.

- 5 3. Inoculation of plates and incubation. 50 μ l of inoculum (*i.e.*, diluted cell suspension) was added to each well. After 20 hours of incubation at 35°C, cell growth was measured in each well and compared to wells with no inoculum.

- 10 4. Results. Figure 15 shows the minimum inhibitory concentration (MIC) of novobiocin, as a function of arabinose concentration, for the P_{BAD} -*gyrB* strain VECO2083. The results show that the MICs of novobiocin, an inhibitor of gyrase subunit B, are arabinose-dependent. Figure 15 also shows that the susceptibility of VECO2083 to compounds that do not target the product of the *gyrB* gene, such as fosfomycin and ciprofloxacin, did not vary with arabinose concentration.

Table 7: Oligonucleotides used for PCR

Oligo	Sequence (5' -> 3')	SEQ ID NO
DYV-157	GCATGCCATGGTTTCAGTTTTGCAAGTGTTAC	27
DYV-158	CGAAGATCTTTAGTTCTTATCCTTAAGC	28
DYV-155	GCGGAGCTCGCAGACTGGCAGCCAGTCG	29
DYV-156	TTGGCGCGCCTCCAGAGATGTGTTTCAGG	30
DYV-095	GCATGCCATGGCAATCAGTCTGATTGCGGCGTTAGC	31
DYV-096	CGAAGATCTTTACCGCCGCTCCAGAATCTCAAAGC	32
DYV-093	GCGGAGCTCGGCGATGCCACGCGGATGG	33
DYV-094	GCTTGGCGCGCCAACGAGTCCACGCTCTCTCC	34
DYV-087	GGTATACCATATGCGAGCTCCAGGCGCGCCTGCAGGA ATTCGATATCAAGC	35
DYV-088	TGCTCTAGAGCCATATGTTCCGCTAGCTTCACGCTGCC	36
DYV-069	GCACCGGAATTCCCGGGTCAGCCAGTCTAACTGCGAA AGCG	37
DYV-163	CCTCGACGGTATCGATAAGC	38
DYV-107	TAGCGGATCCTACCTGACGC	39
DYV-218	CGGGATCCGCGAAGAGTACCAGTACACC	40
DYV-099	GCATGCCATGGCATCGAATTCTTATGACTCCTCC	41
DYV-204	GTCCGATCGTTAAATATCGATATTCGCCGC	42
DYV-097	GCGGAGCTCAGCGATTGCTCAAGCAGCG	43
DYV-098	GCTTGGCGCGCCTCTCGCTCATTATACTTGGG	44
DYV-211	TCAGCGGCCGCCAGCGTGCAGATTGAAGATGC	45
DYV-214	TGACTCGAGCCGTGTAGTAGCTGATATCACGG	46
VCJ005	CCACCATAATTGACGAACGC	47
VCJ007	GTCTTCGGTACGGTCATGGTG	48

Example 11: Construction and properties of a hypersusceptible *E. coli* strain

This example describes the construction of a strain of *E. coli*, VECO2068, with an essential gene, *def*, under P_{BAD} control, and with a deletion in the *tolC* gene. Because the essential *def* gene is under P_{BAD} control, the susceptibility of VECO2068 to inhibitors of the *def* gene product depends on the concentration of arabinose in the growth medium. Mutants in *tolC* are hypersusceptible to many compounds, because *tolC* encodes an outer membrane protein, which can serve as a component of an efflux pump. Thus, the threshold for susceptibility to compounds which interact with the *def* gene product is lowered in a *tolC* mutant, compared to wild-type. Because of its heightened susceptibility, the *tolC*/ P_{BAD} -*def* strain can be used for detecting compounds that otherwise would not have been identified as inhibitors of a strain that is wild-type for *tolC*.

A. Construction of a *tolC* deletion in an *E. coli* strain containing a P_{BAD} -*def* transcriptional fusion

The *tolC* gene was PCR amplified from *E. coli* strain VECO1004 using primers VCJ005 (SEQ ID NO: 47, Table 7) and VCJ007 (SEQ ID NO: 48, Table 7). The 2.7 kb PCR product was blunt-end cloned into pUC18 creating pCH12. A 700 bp internal deletion of *tolC* was created by digestion of pCH12 with the compatible enzymes PstI and NsiI, creating pCH13. The 2.0 kb Δ *tolC* fragment was excised from pCH13 by SmaI/EheI digestion and cloned into SmaI digested pKO3 creating pDY92.

The pDY92 plasmid was used to introduce the *tolC* deletion mutation (Δ *tolC*) into the chromosome of VECO2065 (an *E. coli* strain containing a chromosomal P_{BAD} -*def* fusion) via the selection /counter-selection procedure previously described for other suicide vector constructs. See Examples 8-10 Transformed cells were screened for successful integration of the Δ *tolC* mutation by plating on LB + 0.2% arabinose, and replica-plating onto MacConkey agar, which does not support growth of Δ *tolC* mutants. Confirmation of Δ *tolC* integration in MacConkey-sensitive clones was verified by PCR with oligonucleotides VCJ005 (SEQ ID NO: 47, Table 7) and VCJ007 (SEQ ID

NO: 48, Table 7). A $\Delta tolC$ strain containing the $P_{BAD-def}$ fusion, VECO2068, was selected for susceptibility testing.

B. Susceptibility of VECO2068 to VRC483 and other antimicrobial agents

An experiment was conducted using VECO2068 ($\Delta tolC$, $P_{BAD-def}$) and a parent strain (VECO2066) containing a deleted $tolC$ gene and lacking the $P_{BAD-def}$ fusion, to compare their susceptibility to VRC483 over a range of arabinose concentrations. VRC483 is a compound with antibacterial activity that targets the def gene product. This compound was identified in a deformylase screen at Versicor and the IC_{50} is 11 nM for *E. coli* deformylase. Deformylase activity was measured as described in Example 8 (Rajagopalan *et al.*, *supra*).

1. Preparation of inoculum. Cells were grown overnight in 5 ml of LB supplemented with 0.1 % arabinose, on a rotary shaker at 35°C and 200 rpm. 100 μ l of overnight culture was collected, centrifuged at 14,000 rpm for 5 min at room temperature, and the cell pellet was suspended in 1 ml of medium with no added arabinose. The cell suspension was diluted 1:1000 in medium and used as inoculum.

2. Preparation of 96 well plates. Checkerboard assays were performed in 96-well microtiter plates, in which arabinose concentration was varied in the first dimension, and concentration of antimicrobial compound in the second. The concentration of antimicrobial varied by two-fold between rows. Dilutions were performed in medium supplemented with different concentrations of arabinose, or medium lacking arabinose. A control row lacking antimicrobial, and a control column lacking arabinose, were also included. Total volume in each well was 50 μ l.

3. Inoculation of plates and incubation. 50 μ l of inoculum (*i.e.*, diluted cell suspension) was added to each well. After 24 hours of incubation at 35°C, cell growth was measured in each well and compared to wells with no inoculum.

4. Results. Figure 16 shows the minimum inhibitory concentration (MIC) of VRC483, as a function of arabinose concentration, for the $tolC/P_{BAD-def}$ strain VECO2068. The results show that the MIC of VRC483, a deformylase inhibitor, for VECO2068 is dependent on the concentration of arabinose in the growth

medium. The parent wild type strain was not susceptible to the antibiotic in the range tested. Comparison to Figure 13 (Example 8) indicates that susceptibility to VRC483 occurs at lower arabinose concentrations, as predicted. Figure 16 also shows that the susceptibility of VECO2068 to compounds that do not target the *def* gene, such as fosfomycin and ciprofloxacin, did not vary with arabinose concentration.

Example 12: Construction and properties of a *S. pneumoniae* strain containing a P_{AGA} -*def* transcriptional fusion

A. Construction of P_{AGA} -*def* transcriptional fusions in *S. pneumoniae*

A DNA sequence containing *rafR* and P_{AGA} (*aga* promoter) was PCR-amplified from *S. pneumoniae* VSPN3026 chromosomal DNA using oligonucleotides REGAGAEI5' (SEQ ID NO: 20, Table 6) and REGAGANB3' (SEQ ID NO: 21, Table 6) and cloned as an EcoRI/NdeI fragment into the integration vector pR326 (Claverys *et al.*, *supra*), resulting in plasmid pR326RafRPaga.

The first 317 bp of the deformylase gene (*def*) from *S. pneumoniae* VSPN3026 chromosomal DNA were PCR amplified using oligonucleotides MALDEF5' (SEQ ID NO: 49 Table 8) and MALDEF3' (SEQ ID NO: 50 Table 8) and cloned as an NdeI/BamHI fragment into plasmid pR326RafRPaga, resulting in plasmid pR326RPADEF.

Using pR326RPADEF as a template, a DNA sequence containing only P_{AGA} and the *def* fragment was amplified using oligonucleotides Paga5'EI (SEQ ID NO: 24, Table 6) and MALDEF3' (SEQ ID NO: 50, Table 8) and cloned into the T-tailed pGEM-T Easy Vector (Promega Corporation, Madison, WI). The construct was digested with EcoRI and the P_{AGA} -*def* containing fragment was cloned into the integration vector pR326 to create pR326Pagadef. This plasmid was used to transform VSPN3026, grown in C+Y without sucrose and supplemented with different raffinose concentrations (two-fold dilutions from 2% to 0.008 %). Transformants were used to inoculate tubes containing 2 ml C+Y medium supplemented with chloramphenicol (2.5 µg/ml) and different raffinose

concentrations (1% to 0.041%), and incubated at 37°C overnight. The overnight culture was used to inoculate (10 µl per well) a 96 well microtiter plate containing 200 µl C+Y with different raffinose concentrations, ranging from 1% to 0.041% (see above,) and incubated at 37°C in a CO₂ incubator with 5% CO₂. Cultures were plated on TSA sheep-blood agar plates containing chloramphenicol (2.5 µg/ml) and 0.2% raffinose. The plates were incubated overnight at 35°C and a single colony was picked and transferred to C+Y medium supplemented with chloramphenicol (2.5 µg/ml) and raffinose (0.03%). The resulting *S. pneumoniae* strain, VSPN3044, carries an insertion at the *def* locus in the chromosome, which was verified by PCR using the primers Paga100 (SEQ ID NO: 25, Table 6) and DEF3'Bam (SEQ ID NO: 51, Table 8). The strain carries a truncated 316 bp *def* gene under natural promoter control and a full-length *def* gene under *P_{AGA}* control.

Table 8: Oligonucleotides used in *P_{AGA}* construction and mutant characterization

Oligo	Sequence (5' -> 3')	SEQ ID NO
MALDEF5'	GGGGAATTCCATATGTCTGCAATAGAACGTATTAC	49
MALDEF3'	CCGCGGATCCAAATCGTAGGCTTCCTGTGG	50
DEF3'Bam	GGCGCGGATCCTTAAGCTTCGATTCTGTAAACCATACCTG	51

B. Susceptibility of VSPN3044 to VRC483, vancomycin, and erythromycin

An experiment was conducted using the *P_{AGA-def}* strain VSPN3044 and its parent strain (VSPN3026), to compare their susceptibility to VRC483 as a function of raffinose concentration. Susceptibility of VSPN 3044 to vancomycin and erythromycin at different raffinose concentrations was also tested. VRC483 is a compound with antibacterial activity that targets the *def* gene product. This compound was identified in a deformylase screen at Versicor and the IC₅₀ is 11 nM for *E. coli* deformylase. Deformylase activity was measured as described in Example 8 (Rajagopalan *et al.*, *supra*).

1. Preparation of inoculum. VSPN3044 and VSPN3026 were grown for 4 hours at 37°C in 5 ml C+Y lacking sucrose and supplemented with 0.03 % raffinose. Cells were grown to an OD of 0.2 at 600 nm. Aliquots of 200 µl were frozen at -70°C as a 15% glycerol solution. When needed for inoculation, frozen stock was melted, centrifuged, and the supernatant discarded. The cell pellet was resuspended in 1 ml of C+Y with glucose, diluted 1:1000 into the same medium (chloramphenicol was added to the VSPN3044 medium) and used as inoculum.

2. Preparation of 96 well plates. Checkerboard assays were performed in 96-well microtiter plates, in which raffinose concentration was varied in the first dimension, and concentration of antimicrobial compound (VRC483, vancomycin, or erythromycin) in the second. The concentration of antimicrobial varied by two-fold between rows. Dilutions were performed in C+Y supplemented with different concentrations of raffinose, or C+Y lacking raffinose. A control row lacking antimicrobial, and a control column lacking raffinose, were also included. Total volume in each well was 50 µl.

3. Inoculation of plates and incubation. 50 µl of inoculum (*i.e.*, diluted cell suspension) was added to each well. After 20 hours of incubation at 35°C, cell growth was measured in each well and compared to wells with no inoculum.

4. Results. Figure 17 shows the minimum inhibitory concentration (MIC) of VRC483, as a function of raffinose concentration, for the *P_{AGA-def}* strain VSPN3044, compared to the parent wild type strain VSPN 3026. The results show that, for VSPN3044, the MIC of VRC 483, a deformylase inhibitor, is dependent on the concentration of raffinose in the growth medium. MIC values for the wild-type strain are not dependent on raffinose concentration. Figure 17 also shows that the susceptibility of VSPN 3044 to erythromycin and vancomycin, which do not target the *def* gene, did not vary with raffinose concentration.

Example 13: Construction and properties of a strain containing a *P_{BAD-lpxC}* transcriptional fusion and a *tolC* deletion

This example demonstrates the construction and properties of a fusion in which the gene of interest is inserted downstream of the *P_{BAD}* promoter at the

normal chromosomal location of P_{BAD} . In this way, potential polar effects on genes downstream from the gene of interest are avoided.

A. Construction of an *E. coli* $\Delta tolC$ strain with a P_{BAD} -*lpxC* transcriptional fusion

5 The product of the *lpxC* gene is the enzyme UDP-3-O-(R-3-hydroxymyristoyl)-N-acetylglucosamine deacetylase, which plays a major role in lipopolysaccharide synthesis in Gram-negative bacteria. A DNA sequence containing the full-length *lpxC* gene was PCR-amplified from *E. coli* strain MG1655 chromosomal DNA using oligonucleotides DYV-240 (SEQ ID NO: 56)
10 and DYV-241 (SEQ ID NO: 57, Table 9), and cloned as an NcoI/BglII fragment into pNR41 creating pNR43. pNR41 contains two regions of homology with the *araBAD* locus on the chromosome. One region contains an optimized P_{BAD} promoter and approximately 500 bp of upstream DNA corresponding to the *araC* gene, the second region contains 600 bp of an internal fragment of the *araD* gene.
15 The *araC*- P_{BAD} -*lpxC*-*araD* cassette was excised from pNR43 as an XmaI/Sall fragment and cloned into XmaI/Sall digested pKO3, thus creating pNR48.

 pNR48 was transformed in *E. coli* strain MG1655. Transformants were selected on LB plates supplemented with chloramphenicol (25 μ g/ml) at 30°C. A number of transformants were streaked onto LB plates supplemented with
20 chloramphenicol (25 μ g/ml) and incubated at 43°C overnight. Isolated colonies were then restreaked at 43°C onto LB plates supplemented with chloramphenicol (25 μ g/ml). Isolated colonies were next streaked onto LB plates and incubated at 37°C overnight. Isolated colonies were then streaked onto LB plates supplemented with sucrose (6%) and incubated at 37°C overnight to select for
25 sucrose-resistant recombinants. NaCl was omitted from LB plates during sucrose resistance selection. Sucrose-resistant recombinants were screened for the inability to ferment arabinose on MacConkey agar plates supplemented with 0.4% arabinose and scored for chloramphenicol sensitivity. Chloramphenicol-sensitive clones that were deficient in arabinose utilization were candidates for successful
30 integration of the *lpxC* gene into the *araBAD* operon. One clone, VECO2520

(*araC*-P_{BAD}-*lpxC*), was verified by checking the chromosomal junctions with PCR primer pair DYV-246/DYV-249 (SEQ ID NOS: 58 and 59, Table 9).

Having placed the *lpxC* gene under arabinose control at the *araBAD* locus, the next step was to delete the endogenous *lpxC* gene from its normal
5 chromosomal context. An in frame deletion of *lpxC*, which resides in a dicistronic operon with the essential gene *secA*, was made using crossover PCR. Link *et al.* (1997) *J. Bacteriol.* 179:6228-6237. The crossover PCR reaction created a 1.2 kb product consisting of 600 bp fragments of DNA to the left and right of the sequence targeted for deletion. The four primers used for crossover
10 PCR amplification were DYV-224 (SEQ ID NO: 52), DYV-225 (SEQ ID NO: 53), DYV-226 (SEQ ID NO: 54) and DYV-227 (SEQ ID NO: 55, Table 9). The resulting PCR product was digested with BamHI and cloned into BamHI-digested pKO3, creating pNR36.

Plasmid pNR36 was transformed in *E. coli* strain VECO2520.
15 Transformants were selected on LB plates supplemented with 0.2% arabinose and chloramphenicol (25 µg/ml) at 30°C. A number of transformants were streaked onto LB plates supplemented with 0.2% arabinose and chloramphenicol (25 µg/ml) and incubated at 43°C overnight. Isolated colonies were then restreaked at 43°C onto LB plates supplemented with 0.2% arabinose and chloramphenicol (25
20 µg/ml). Isolated colonies were next streaked onto LB plates supplemented with 0.2% arabinose and incubated at 37°C overnight. Isolated colonies were then streaked onto LB plates supplemented with 0.2% arabinose and sucrose (6%) and incubated at 37°C overnight to select for sucrose-resistant recombinants. NaCl was omitted from LB plates during sucrose resistance selection. Sucrose-resistant
25 recombinants were screened for arabinose-dependent growth and scored for chloramphenicol sensitivity. Chloramphenicol-sensitive clones that required arabinose for growth were candidates for successful deletion of the *lpxC* gene from its normal chromosomal context. One clone, VECO2522 (*araC*-P_{BAD}-*lpxC*, Δ *lpxC*), was verified by checking the chromosomal junctions with PCR primer
30 pairs DYV-224/DYV-227 (SEQ ID NOS: 52 and 55, Table 9).

The *tolC* gene was PCR-amplified from *E. coli* strain VECO1004 using primers VCJ005 (SEQ ID NO: 47, Table 7) and VCJ007 (SEQ ID NO: 48, Table 7). The 2.7 kb PCR product was blunt-end cloned into pUC18 creating pCH12. A 700 bp internal deletion of *tolC* was created by digestion of pCH12 with the compatible enzymes PstI and NsiI, creating pCH13. The 2.0 kb $\Delta tolC$ fragment was excised from pCH13 with SmaI-EheI and cloned into SmaI digested pKO3, creating pDY92.

The $\Delta tolC$ mutation was introduced onto VECO2522 and *E. coli* MJ1655 with pDY92 via the selection/counter-selection procedure previously described for other suicide vector constructs. Successful integration of the $\Delta tolC$ mutation was screened on MacConkey agar which does not support growth of $\Delta tolC$ mutants. Confirmation of $\Delta tolC$ integration in MacConkey-sensitive clones was verified by PCR with oligonucleotides VCJ005 (SEQ ID NO: 47, Table 7) and VCJ007 (SEQ ID NO: 48, Table 7). VECO2524 is an *araC*-P_{BAD}-*lpxC*, $\Delta lpxC$, $\Delta tolC$ mutant and VECO2526 is a $\Delta tolC$ mutant. These strains were used for further experiments.

B. Susceptibility of VECO2524 (P_{BAD}-*lpxC*) to L159692 and other antimicrobial agents

An experiment was conducted using the VECO2524 strain and the *tolC* isogenic strain (VECO2526), to compare their susceptibility to L159692 within a range of arabinose concentrations. L159692 is an antibacterial compound that targets the *lpxC* gene product. Onishi *et al.* (1996) *Science* 274:980-982. The susceptibility of VECO2526 to other unrelated antibiotics, linezolid and erythromycin, was also tested.

1. Preparation of inoculum. Cells were grown overnight in 5 ml of LB supplemented with 0.1 % arabinose, on a rotary shaker at 35°C and 200 rpm. 100 μ l of overnight culture was collected, centrifuged for 5 min. at room temperature at 14,000 rpm, and the cell pellet was suspended in 1 ml of medium with no added arabinose. The cell suspension was diluted 1:1000 in medium and used as inoculum.

2. Preparation of 96 well plates. Checkerboard assays were performed in 96-well microtiter plates, in which arabinose concentration was varied in the first dimension, and antimicrobial compound in the second. Antimicrobial concentration varied by two-fold between rows. Dilutions were performed in medium supplemented with different concentrations of arabinose or lacking arabinose. A control row lacking antimicrobial, and a control column lacking arabinose, were also included. Total volume in each well was 50 μ l.

3. Inoculation of plates and incubation. 50 μ l of inoculum (*i.e.*, diluted cell suspension) was added to each well. After 20 hours of incubation at 35°C, cell growth was measured in each well and compared to wells with no inoculum.

4. Results. Figure 18 shows the minimum inhibitory concentration (MIC) of L159692, linezolid, and erythromycin, as a function of arabinose concentration, for the P_{BAD} -*lpxC* strain. The susceptibility of the isogenic *tolC* strain, VECO2526, to any of the compounds tested was not influenced by the amount of inducer in the medium.

Table 9: Oligonucleotides used for PCR in the construction of P_{BAD} -*lpxC* fusions

Oligo	Sequence	SEQ ID NO
DYV-224	TCGGATCCGGCTACGCAATGATGGGTTTC	52
DYV-225	CCCATCCACTAAACTTAAACATGTCCTTTGTTTGATCATCG	53
DYV-226	TGTTTAAGTTTAGTGGATGGGTTGGCCTTCAAAGCGCCTTCA	54
DYV-227	GTGGATCCGTAATGCAAGATCTTGCGC	55
DYV-240	GGTTCATGGCAATCAAACAAAGGACACTTAAACG	56
DYV-241	GTCAGATCTTTATGCCAGTACAGCTGAAGG	57
DYV-246	GACCCGGGTGATACCATTGCGGAGCC	58
DYV-249	GAGTCGACGCAGCGTTTGCTGCATATCC	59

Example 14: Regulatory properties of the *S. pneumoniae* *raf* gene cluster

The regulation of the *S. pneumoniae* *raf* operons by various sugars, including raffinose, was investigated, using α -galactosidase activity as a reporter. The *aga* gene exhibits sequence homology to other prokaryotic α -galactosidases,

and this example shows that α -galactosidase activity is encoded by *aga* in *S. pneumoniae*. Hence, the *S. pneumoniae aga* gene, regulated by its promoter P_{AGA} , serves as a naturally-occurring reporter gene for use in the study of induction and regulation of the *S. pneumoniae raf* gene cluster, and can also be used as a reporter gene for the analysis of other potential regulatory sequences in *S. pneumoniae* and other microorganisms.

Cells and Cell Growth

S. pneumoniae strain VSPN3026 was used as wild-type for the experiments described herein. Cells were grown in C+Y Medium (Tomasz (1970) *J. Bacteriol.* **101**:860-871) containing 0.2% (w/v) sucrose and 0.2% (w/v) glucose, and logarithmic phase cells were frozen in 20% (v/v) glycerol, to be used for inoculation. In experiments to identify inducers of galactosidase activity, C+Y medium containing various sugars was used. All sugars were used at a concentration of 0.2% (w/v). Eight ml of medium was inoculated with 200 μ l of frozen stock cells, and the culture was grown at 37°C. Growth of the culture was measured by absorbance at 600 nm, using a visible spectrophotometer. When A_{600} reached 0.4, cells were used for experiments.

Measurement of α -galactosidase activity

To prepare cell lysates for measurement of enzyme activity, 1.5 ml samples of cell culture were collected and immediately centrifuged for 5 min. at 14,000 rpm to pellet the cells. The supernatant was discarded and the pellet was resuspended in 0.1 ml of 100 mM sodium phosphate buffer, pH 7.5 containing 0.25% Triton X-100. This mixture was incubated at 37°C for 10 min to lyse the cells.

Alpha-galactosidase activity was measured in a buffer containing 100 mM sodium phosphate, 1 mM $MgCl_2$, 45 mM β -mercaptoethanol, pH 7.5, containing *p*-nitrophenyl- α -D-galactopyranoside (Sigma Chemical, St. Louis, MO) at a final concentration of 0.9 mg/ml. The reaction was initiated by addition of 10 μ l cell lysate to 90 μ l of reaction buffer and the reaction mixture was incubated at 25°C. Enzymatic activity was monitored by measuring absorbance at 405 nm. A_{405} measurements were taken every 30 sec for 30 min using a Spectramax 250

microtiter plate reader (Molecular Devices, Sunnyvale, CA). Specific activity was calculated using a *p*-nitrophenol standard.

Results are shown in Table 10. As can be seen, low basal levels of α -galactosidase activity were observed in lysates from cells grown with sugars other than raffinose as a sole carbon source. However, a 200-1,000-fold induction of α -galactosidase activity was observed in cells grown on raffinose. Combinations of raffinose and a second sugar gave enzyme levels that were 16-500-fold greater than those obtained with the second sugar alone. Thus, by adjusting the concentration of sugar and/or the combination of sugars in the medium, expression of coding sequences regulated by *raf* regulatory sequences can be modulated over an approximately 1,000-fold range.

Table 10: Galactosidase activity measured in lysates from cells grown on different sugars

Sugar*	Specific Activity (nmol <i>p</i> -nitrophenol/min/mg protein)
glucose	12
fructose	20
sucrose	24
galactose	30
lactose	17
maltose	7
raffinose	7,099
fructose + raffinose	4,681
sucrose + raffinose	382
galactose + raffinose	9,714
lactose + raffinose	8,258
maltose + raffinose	3,580

*All sugars are present in the medium at a concentration of 0.2% (w/v)

Construction of mutants in the *aga*, *rafR* and *rafS* genes

To characterize the *raf* regulatory system, gene knockouts in the *aga*, *rafR* and *rafS* genes were constructed by the method of Claverys *et al.* (1995) *Gene* 164:123-128, as follows.

5 A DNA fragment containing a region of the *aga* gene was amplified by polymerase chain reaction (PCR) using oligonucleotides *aga1* and *aga2* as primers. See Table 11 for the sequences of oligonucleotides. This generated a 320 bp amplification product. The amplified sequence was ligated into pGEM-T Easy (Promega, Madison, WI). The resulting construct was digested with EcoRI, to release an approximately 339 bp fragment containing *aga* sequences. This fragment was inserted into the EcoRI site of pR 326 (Claverys *et al.*, *supra*) to create pR326AGAKO. This plasmid was used to transform VSPN3026 to construct a *S. pneumoniae* strain, VSPN3037, with an insertion of pR326 sequence in the chromosomal *aga* gene, thereby inactivating the *aga* gene.

15 A DNA fragment containing an internal portion of the *rafR* gene was PCR-amplified using oligonucleotides *rafR1* and *rafR2* as primers. See Table 11 for sequences. This generated a 449 bp amplification product. The amplified sequence was ligated into pCRII (Invitrogen, Carlsbad, CA). The resulting construct was digested with EcoRI, and an approximately 440 bp *rafR*-containing fragment was inserted into the EcoRI site of pR 326 to create pR326RAFRKO. This plasmid was used to transform VSPN3026 to construct a *S. pneumoniae* strain, VSPN3038, with an insertion of pR326 sequence in the chromosomal *rafR* gene, thereby inactivating the *rafR* gene.

25 A DNA fragment containing an internal portion of the *rafS* gene was PCR-amplified using oligonucleotides *rafS1* and *rafS2* as primers. See Table 11 for sequences. This generated a 454 bp amplification product, which was ligated into pCRII (Invitrogen, Carlsbad, CA). The resulting construct was digested with EcoRI and an approximately 445 bp *rafS*-containing fragment was obtained, which was inserted into the EcoRI site of pR 326 to create pR 326RAFSKO. This plasmid was used to transform VSPN3026 to construct a *S. pneumoniae* strain,

30

VSPN3039, with an insertion of pR326 sequence in the chromosomal *rafS* gene, thereby inactivating the *rafS* gene.

Table 11: Sequences of Oligonucleotide used for construction of mutations in the *aga*, *rafR* and *rafS* genes

Oligonucleotide	Sequence (5' → 3')	SEQ ID NO.
aga 1	GCTCAACTTAGTCTGACTTTG	60
aga 2	CAAACACATTCCCAGCATCCTCTG	61
rafR 1	CGCGGATCCTCGAGAAGTTGTCTAGCTCGG	62
rafR 2	CCGGAATTCTAGGAATCACTGGAGGGAAA	63
rafS 1	CCGCGGATCCGCTACAAGTAGTGTGTAGGATGG	64
rafS 2	GCCGGAATTCAATCCTACCAAGCTGTCTACC	65

Characterization of mutants

The *aga*, *rafR* and *rafS* mutant strains were tested for growth and for α -galactosidase activity, when provided with raffinose, sucrose or a mixture of raffinose and sucrose as carbon source. Mutant strains were grown in C+Y medium containing different carbon sources (as indicated in Table 12) and growth was monitored by absorbance of cultures at 600 nm, measured by spectrophotometry. When cultures reached an A_{600} of 0.4, cells were collected and assayed for α -galactosidase activity as described *supra*. Assay results are shown in Table 12.

Strains with a mutation in *aga* were unable to grow on raffinose. The inability of an *aga* mutant strain to grow on raffinose confirmed that mutation had occurred in a gene necessary for raffinose metabolism and, since raffinose is an α -galactoside, is consistent with the inactivation of an α -galactosidase.

The *aga* mutant strains grew when either sucrose or sucrose + raffinose were provided as carbon sources, but exhibited non-measurable levels of α -galactosidase activity under both of these conditions. See Table 12. These results indicate that the α -galactosidase activity observed in wild-type cells grown on raffinose is provided by the product of the *aga* gene. Taken together, the

results indicate that expression of the *aga* gene is activated by raffinose; *i.e.*, raffinose is an inducer of *aga*.

5 A strain harboring a mutation in the *rafR* gene is able to grow on raffinose, but induced levels of α -galactosidase in this strain are seven-fold lower than in wild-type cells. *See* Table 12. Thus, *rafR* function is required for maximal induction of *aga* activity. These results are those expected if the *rafR* gene product acts as an activator of the *aga* gene, and are consistent with the presence, in the amino acid sequence of the *rafR* gene product, of the AraC family signature sequence. They are also consistent with the high degree of homology between the
10 RafR amino acid sequence and the sequences of other transcriptional activator proteins.

Strains with *rafR* mutations grow slowly when raffinose is provided as a sole carbon source, as expected if RafR is an inducer of the *raf* metabolic operon. However, *rafR* mutants also grow more slowly than wild-type when sugars other
15 than raffinose are provided as a carbon source. This suggests that RafR has additional regulatory targets outside the *raf* metabolic operon, and that the RafR protein may have additional regulatory functions beyond those that are related to raffinose metabolism. Products of non-*raf* genes that are regulated by RafR may serve as potential targets for drug discovery.

20 Strains carrying mutations in *rafS* that were grown in the presence of raffinose (*i.e.*, either raffinose alone or raffinose + sucrose) express higher levels of α -galactosidase activity than wild-type cells. These results are consistent with the *rafS* gene product being a negative regulator of *aga* expression.

An alternative interpretation of these results is possible if, for example,
25 inactivation of *rafR* has a polar effect on *rafS*, such that the activity detected in *rafR* mutants reflects absence of both RafR and RafS function. If this were the case, the *rafR* gene product could be an activator, or the combined activity of the *rafR* and *rafS* gene products could provide activator function.

**Table 12: α -galactosidase activity in mutant strains
grown on different carbon sources**

Strain	Specific Activity (nmol <i>p</i> -nitrophenol/min/mg protein)		
	Sucrose	Raffinose	Sucrose + Raffinose
VSPN3026 (wild-type)	4.8	2,538	137
VSPN3037(<i>aga</i> ⁻)	0.1	ND (no growth)	0.1
VSPN3038(<i>rafR</i> ⁻)	3.9	346	32
VSPN3039 (<i>rafS</i> ⁻)	8.3	3,756	403

While the foregoing invention has been described in some detail by way of
5 illustration and example for purposes of clarity of understanding, it will be
apparent to those skilled in the art that various changes and modifications may be
practiced without departing from the spirit of the invention. Therefore the
foregoing descriptions and examples should not be construed as limiting the scope
of the invention.

CLAIMS

What is claimed is:

1. A cell which expresses a gene involved in an essential cellular process, wherein expression of the gene can be regulated over a range of levels,
5 and further wherein the range includes a low basal expression level.
2. A cell according to claim 1, wherein the basal expression level is less than about 50% of wild type.
3. A cell according to claim 1, wherein the basal expression level is insufficient to support cell growth.
- 10 4. A cell according to claim 1, wherein the gene encodes a polypeptide.
5. A cell according to claim 1, wherein the gene encodes an untranslated RNA molecule.
6. A cell according to claim 4, wherein the polypeptide is selected
15 from the group consisting of a structural protein, an enzyme, a receptor, an intracellular signaling molecule and a cellular adhesion molecule.
7. A cell according to claim 1, wherein the cellular process is selected from the group consisting of replication, recombination, DNA repair, transcription, translation, protein processing, protein export, cell wall
20 biosynthesis, cell membrane synthesis, lipid metabolism, protein metabolism, energy metabolism, cell division, drug resistance and virulence.
8. A cell according to claim 1, wherein expression of the gene is regulated by fusion of the gene to a heterologous regulatory element.
9. A cell according to claim 4, wherein expression of the polypeptide
25 is regulated by a heterologous regulatory element that has been fused to a sequence which encodes the polypeptide or a fragment thereof.
10. A cell according to claim 9, wherein the regulatory element is the P_{BAD} promoter.
11. A cell according to claim 10, wherein expression is regulated by
30 adjusting the concentration of L-arabinose or L-ribose in the cellular growth medium.

12. A cell according to claim 11, wherein further regulation is achieved by adjusting the concentration of glucose or other carbon source in the cellular growth medium.

13. A cell according to claim 9, wherein the regulatory element is
5 selected from the group consisting of the *lac* promoter, the *trp* promoter, the *tac* promoter, the *gal* promoter, the *lpp* promoter, the *phoA* promoter, the T7 promoter, the T3 promoter, the SP6 promoter, the λp_R promoter, the λp_L promoter, and the *tet* promoter.

14. A cell according to claim 9, wherein the heterologous regulatory
10 element is regulated by a member of the AraC/XylS family.

15. A cell according to claim 9, wherein the heterologous regulatory element is regulated by a two-component regulatory system.

16. A cell according to claim 1, wherein the cell is a microorganism.

17. A cell according to claim 16, wherein the cell is a prokaryotic cell.

18. A cell according to claim 17, wherein the cell is Gram-positive.

19. A cell according to claim 17, wherein the cell is Gram-negative.

20. A cell according to claim 1, wherein the cell is a eukaryotic cell.

21. A cell according to claim 20, wherein the cell is a yeast cell.

22. A cell according to claim 20, wherein the cell is a fungal cell.

23. A cell according to claim 20, wherein the cell is a plant cell.

24. A cell according to claim 20, wherein the cell is a mammalian cell.

25. A cell according to claim 24, wherein the cell is a human cell.

26. A cell according to claim 25, wherein the cell is malignant.

27. A cell according to claim 26, wherein the cell is resistant to a
25 therapeutic.

28. A cell according to claim 17, wherein the cell is resistant to a therapeutic.

29. A cell according to claim 20, wherein the cell is resistant to a therapeutic.

30. A method for identifying a compound that affects an essential
30 cellular process, the method comprising the steps of:

- (a) providing a cell according to claim 1,
- (b) exposing the cell to the compound; and
- (c) assaying cell viability.

31. The method according to claim 30, wherein the cell is cultured in a liquid medium and the cell is exposed to the compound by addition of the compound to the culture medium.

32. The method according to claim 30, wherein the cell is cultured on a solid medium and the cell is exposed to the compound by application of the compound to the solid medium.

33. The method according to claim 30, wherein cell viability is assayed by measuring cell growth.

34. The method according to claim 33, wherein cell growth is determined by a measurement selected from the group consisting of vital staining, cell counting, light scattering, incorporation of macromolecular precursor, fluorescence-activated cell sorting and reporter gene expression.

35. A method for determining the target of a compound, the method comprising the steps of:

(a) providing a library of cells according to claim 1, wherein, in each member of the library, the expression of a different gene product is regulated, and wherein, in the cells comprising the library, a variety of different gene products are regulated;

- (b) exposing the library to the compound; and
- (c) assaying cell growth;

wherein, if the growth of a member of the library is negatively affected, the gene product regulated in that member is the target.

36. A method for identifying a gene that is involved in an essential cellular process, the method comprising the steps of:

(a) constructing a fusion between a heterologous regulatory element and a coding sequence, wherein the heterologous regulatory element allows expression of the coding sequence over a range of levels, and further wherein the range includes a low basal expression level,

(b) exposing a cell containing the fusion to a concentration of a test compound, and

(c) assaying cell viability in the presence of the test compound.

37. The method according to claim 36, wherein the fusion is
5 constructed *in vitro* and is introduced into a cell.

38. The method according to claim 36, wherein the fusion is constructed *in vivo* within the cell.

39. The method according to claim 36, wherein cell viability is assayed by measuring cell growth.

10 40. The method according to claim 36, wherein viability is assayed at more than one level of expression of the coding sequence.

41. The method according to claim 36, wherein the coding sequence encodes an untranslated RNA molecule

42. The method according to claim 36, wherein the coding sequence
15 encodes a polypeptide or a fragment thereof

43. The method according to claim 36, wherein viability is assayed at more than one concentration of the test compound.

44. The method according to claim 40, wherein viability is assayed at more than one concentration of the test compound.

20 45. The method according to claim 35, wherein the regulatory element is the P_{BAD} promoter.

46. The method according to claim 45, wherein expression is regulated by adjusting the concentration of L-arabinose or L- ribose in the cellular growth medium.

25 47. The method according to claim 46, wherein further regulation is achieved by adjusting the concentration of glucose or other carbon source in the cellular growth medium.

48. The method according to claim 36, wherein the regulatory element is selected from the group consisting of the *lac* promoter, the *trp* promoter, the *gal* promoter, the T7 promoter, the T3 promoter, the SP6 promoter, the λ pr promoter, the λ pL promoter and the *tet* promoter.
30

49. The method according to claim 36, wherein the heterologous regulatory element is regulated by a member of the AraC/XylS family.

50. The method according to claim 36, wherein the heterologous regulatory element is regulated by a two-component regulatory system.

5 51. The method according to claim 36, wherein the basal expression level is less than about 50% of wild type.

52. The method according to claim 42, wherein the coding sequence encodes a polypeptide selected from the group consisting of a structural protein, an enzyme, a receptor, an intracellular signaling molecule and a cellular adhesion
10 molecule.

53. The method according to claim 36, wherein the cellular process is selected from the group consisting of replication, recombination, DNA repair, transcription, translation, protein processing, protein export, cell wall biosynthesis, cell membrane synthesis, lipid metabolism, protein metabolism,
15 energy metabolism, cell division, drug resistance and virulence.

54. The method according to claim 36, wherein the cell is cultured in a liquid medium and the cell is exposed to the compound by addition of the compound to the culture medium.

55. The method according to claim 36, wherein the cell is cultured on a
20 solid medium and the cell is exposed to the compound by application of the compound to the solid medium.

56. The method according to claim 36, wherein cell growth is determined by a measurement selected from the group consisting of vital staining, cell counting, light scattering, incorporation of macromolecular precursor,
25 fluorescence-activated cell sorting and reporter gene expression.

57. A method for identifying a gene responsible for conferring sensitivity to a test compound, the method comprising identifying a gene according to the method of claim 36.

58. A method for identifying a gene responsible for conferring
30 resistance to an antibiotic, the method comprising identifying a gene according to

claim 36 wherein the test compound is an antibiotic and the essential cellular function is antibiotic resistance.

59. A method for identifying a gene responsible for virulence, using the cell of claim 1, wherein the essential cellular process is involved in virulence.

5 60. A method for identifying a polypeptide that is involved in an essential cellular process, the method comprising identifying a gene according to claim 36 and determining the polypeptide from the sequence of the gene.

61. A method for identifying an RNA that is involved in an essential cellular process, the method comprising identifying a gene according to claim 36 and determining the identity of the RNA from the sequence of the gene.

62. A cell according to claim 9, wherein the heterologous regulatory element is selected from the group consisting of the *mal Px* promoter and the *mal Pm* promoter.

63. A cell according to claim 62, wherein expression is regulated by adjusting the concentration of maltose in the growth medium.

64. A cell according to claim 63, wherein the cell is grown in a minimal medium.

65. The method according to claim 36, wherein the heterologous regulatory element is selected from the group consisting of the *mal Px* promoter and the *mal Pm* promoter.

66. The method according to claim 65, wherein expression is regulated by adjusting the concentration of maltose in the growth medium.

67. The method according to claim 66, wherein the cell is grown in a minimal medium.

25 68. The modified *mal Px* promoter according to Figure 1, wherein the sequence GGA at approximately -80 is converted to GCG.

69. A cell according to claim 9, wherein the heterologous regulatory element is selected from the group consisting of *raf P_{AGA}*, *raf P_R* and *raf P_E*.

30 70. A cell according to claim 69, wherein expression is regulated by adjusting the concentration of raffinose in the growth medium.

71. A cell according to claim 69, wherein expression is regulated by adjusting the concentration of sucrose in the growth medium.

72. The method according to claim 36, wherein the heterologous regulatory element is selected from the group consisting of *raf P_{AGA}*, *raf P_R* and
5 *raf P_E*.

73. The method according to claim 72 wherein the heterologous regulatory element is regulated by adjusting the concentration of raffinose in the growth medium.

74. The method according to claim 72 wherein the heterologous
10 regulatory element is regulated by adjusting the concentration of sucrose in the growth medium.

75. The method according to claim 30, wherein the cell is hypersusceptible to the compound.

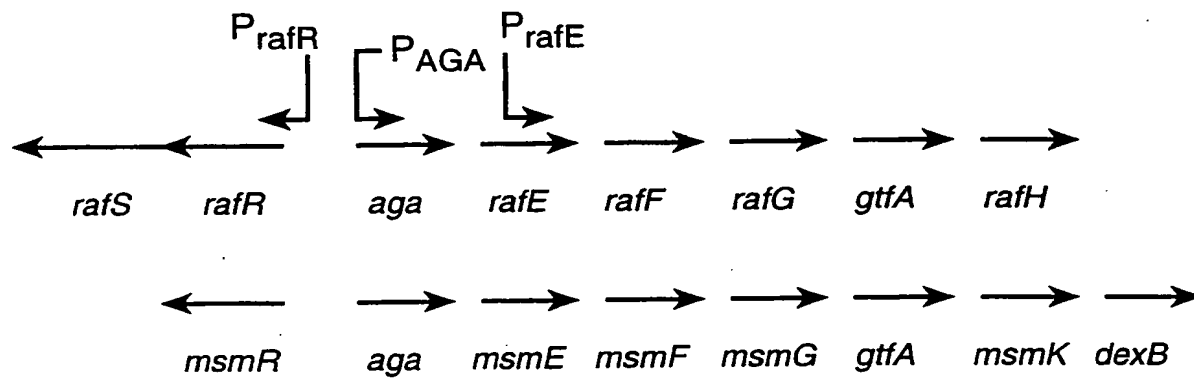
76. The method according to claim 75, wherein hypersusceptibility is
15 due to a mutation in a cellular gene.

77. The method according to claim 76, wherein the cellular gene encodes a component of an efflux pump.

[illegible]

FIG. 1

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**FIG. 2**

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ATTTTACTTC CAACTATTGA GAGAAATTTT GCCACTATTT AGCCAGATTT CTTTTCCGTT 60
ATCACATTGA ACTAAAAGTT TTCCATTTTC TGAGATGTCT TTAGCAAGTC CCTTG TAGTC 120
TTTTTGCTCT AGTGTGAAAG TGACTTCTTT TCCTAGAATG AATGACTGTT TTTTGTATAG 180
GTATAATAGC TCTTCTGCTG GTGTTTCGAA GAAAGCACGC CAGATTTCTA TGATCAATTC 240
ATTCCTTGTT ATAGGAGCTG TAGCTTTAAA TAAGCTGGCA GCTTTTTCTT TTAATTCCTG 300
AGGGAAGTCT TTAATAGTGA AATTGATCCT ACTCCAATAA TGATATCTGT GACTAAGCCA 360
GTTTCTACAG AGGTCATTGC TTCAGTAAGG ATTCCTCCAA TTTTATGATT GTTTAGATAG 420
ATATCATTGA CCCATTTTAT ATCGACATCT ATTAAAGTTA GGTTCTTAAT GGCTTTGTAG 480
ACAGCTCCAG CTACAAAGTAG TGTGTAGGAT GGTAATTTGT CATAGGGGAG ATTTGGTTTA 540
AGATGGAGTG TCATATAAAT TCCACCTTGT GGTGAGTAGA AGGAACGTTG AAAACGGCCT 600
CGGCCTGCTG TTTGATAGGA AGCTAGATAG AGAGTATTTG CTTCATGGCC TAAATCAATT 660
GCTTCTTTTG CATCTAGTTG TGTGATTTT GTTTCGGGT TAAAGCTGAC TTTAATTGGA 720
AGATTTTCTT CTAGAATCTC TGAAGAATA AGGTCACCAT TCATTAGTTT ATATCCTCTA 780
TTTTTGATAC TATTAATTTT AATGCCTTCT TGTTCTAGTC GCTTGATAGC TTTCCAAATT 840
GATGTTCCGC TTAGGGATAG TTTTCTGCG ATTTTTTCTC CGCTGATATA GTCGGTTTCT 900
TTAGATAGGA TTTGGTAGAC AGCTTGGTAG GATTTTCATAA TGTTGCCCTT CTCACTAGTT 960
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CTTTCGAAAA ATGGAGTGGA TCTGAAAAAC CTACCGAGTA TGCAATTACC TTGATGGACT 1080
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FIG. 3-1

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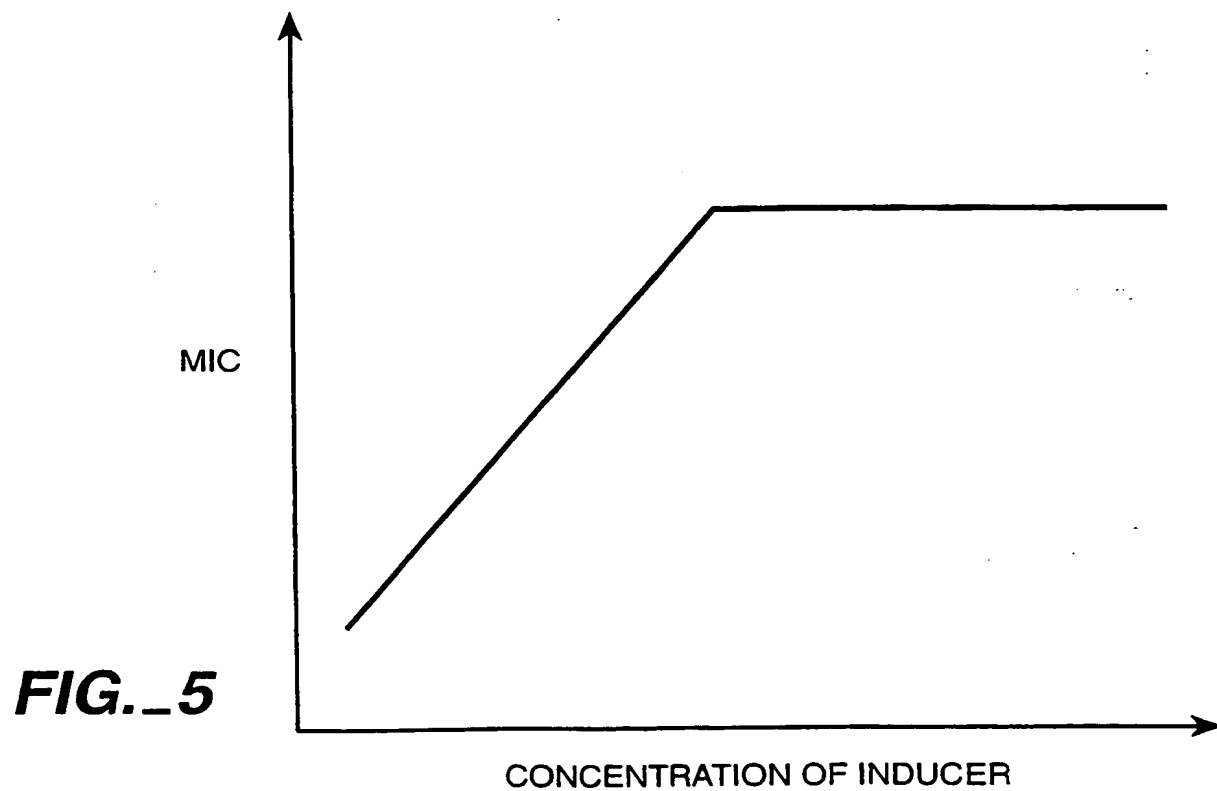
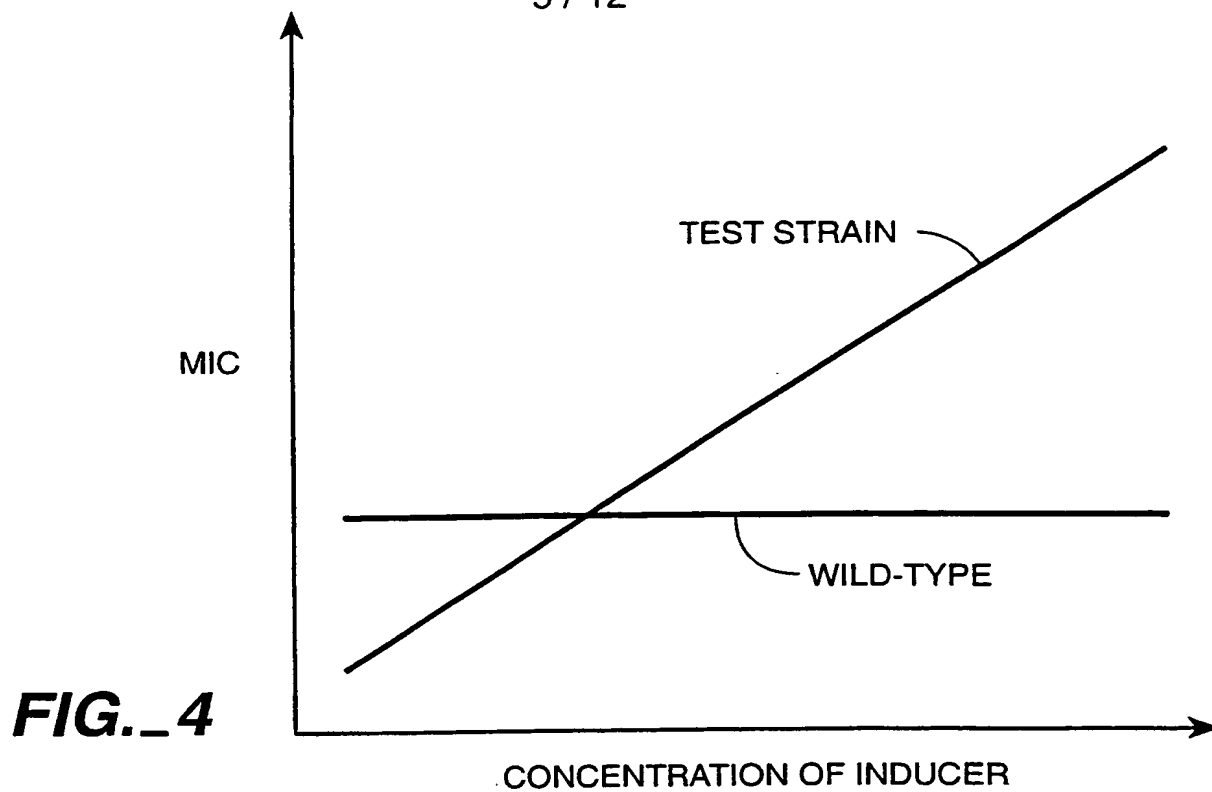
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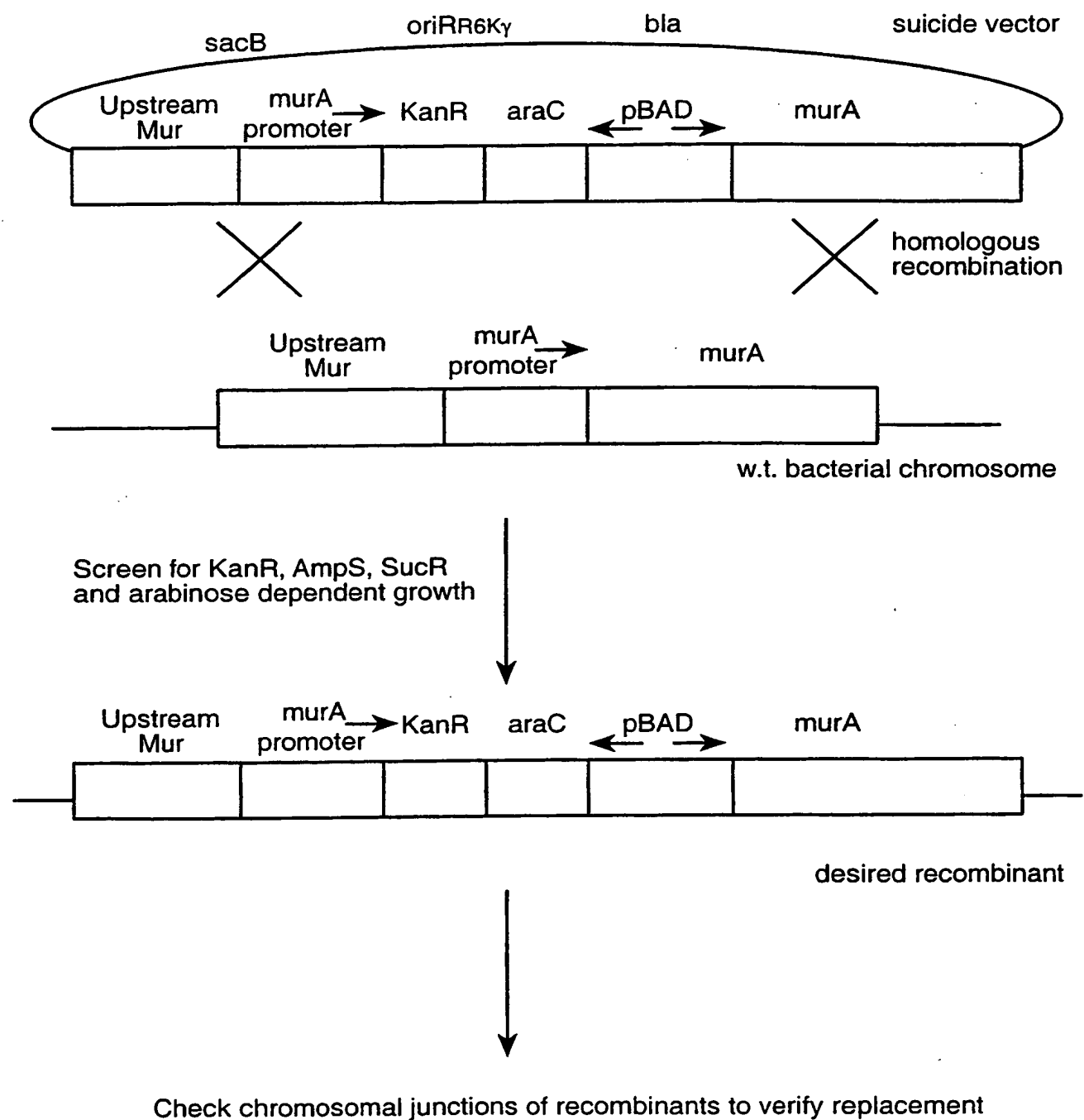
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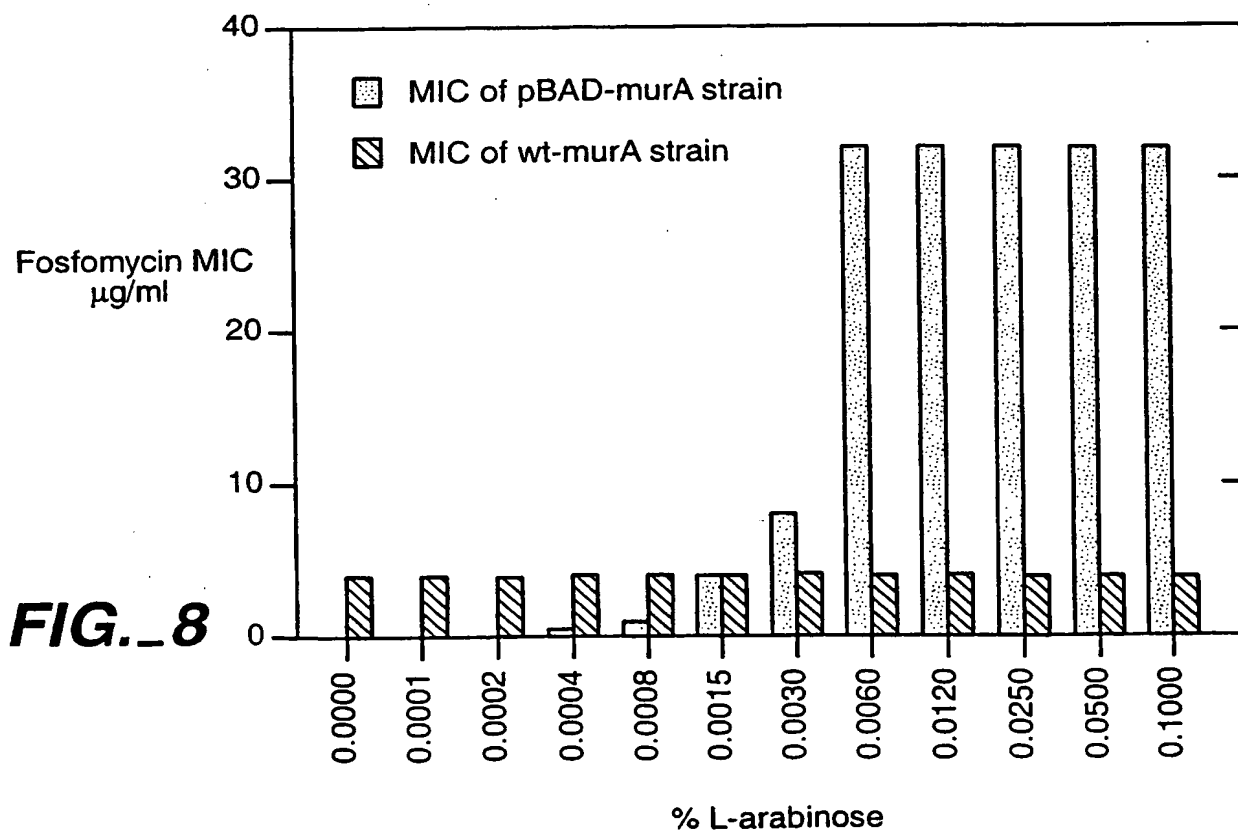
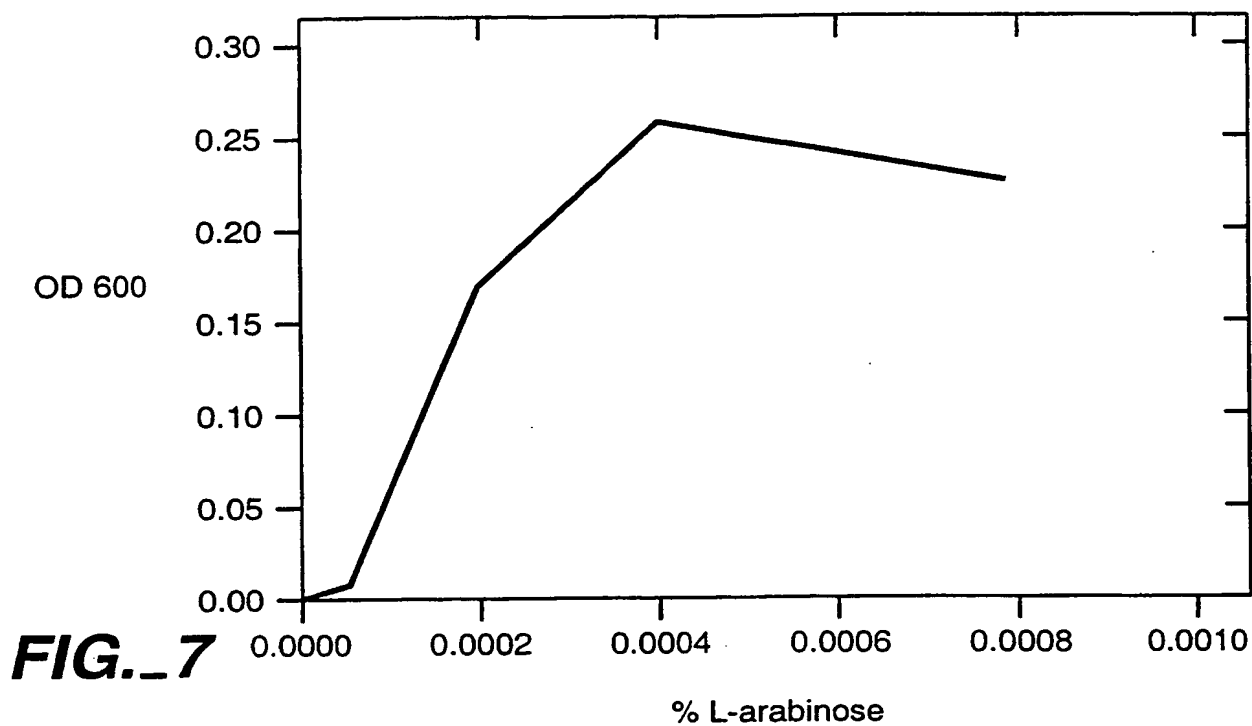
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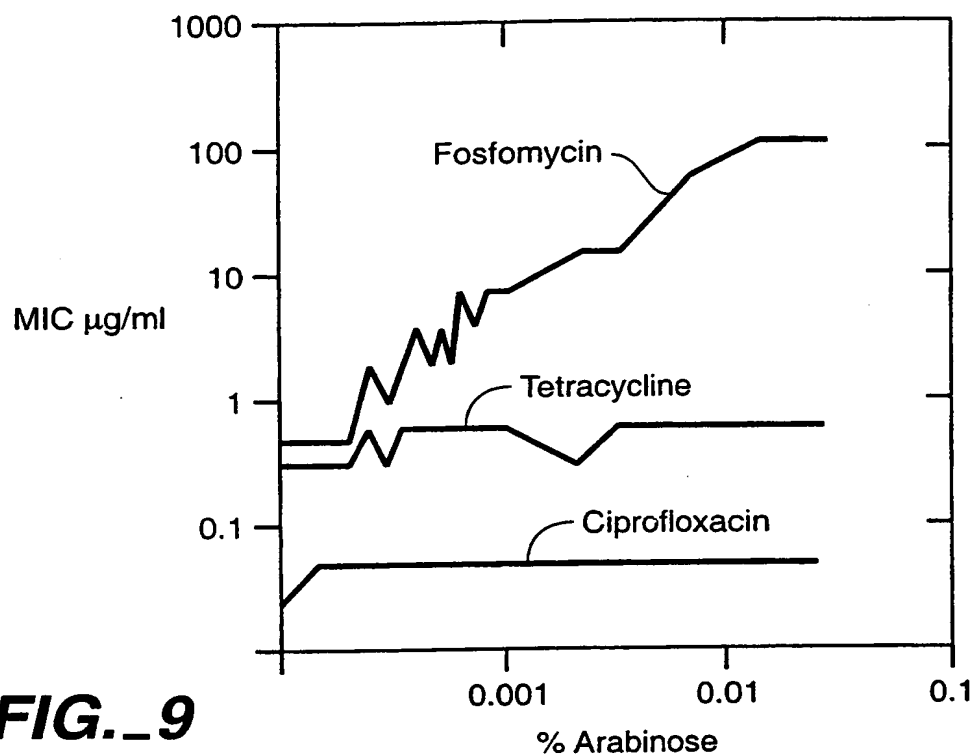
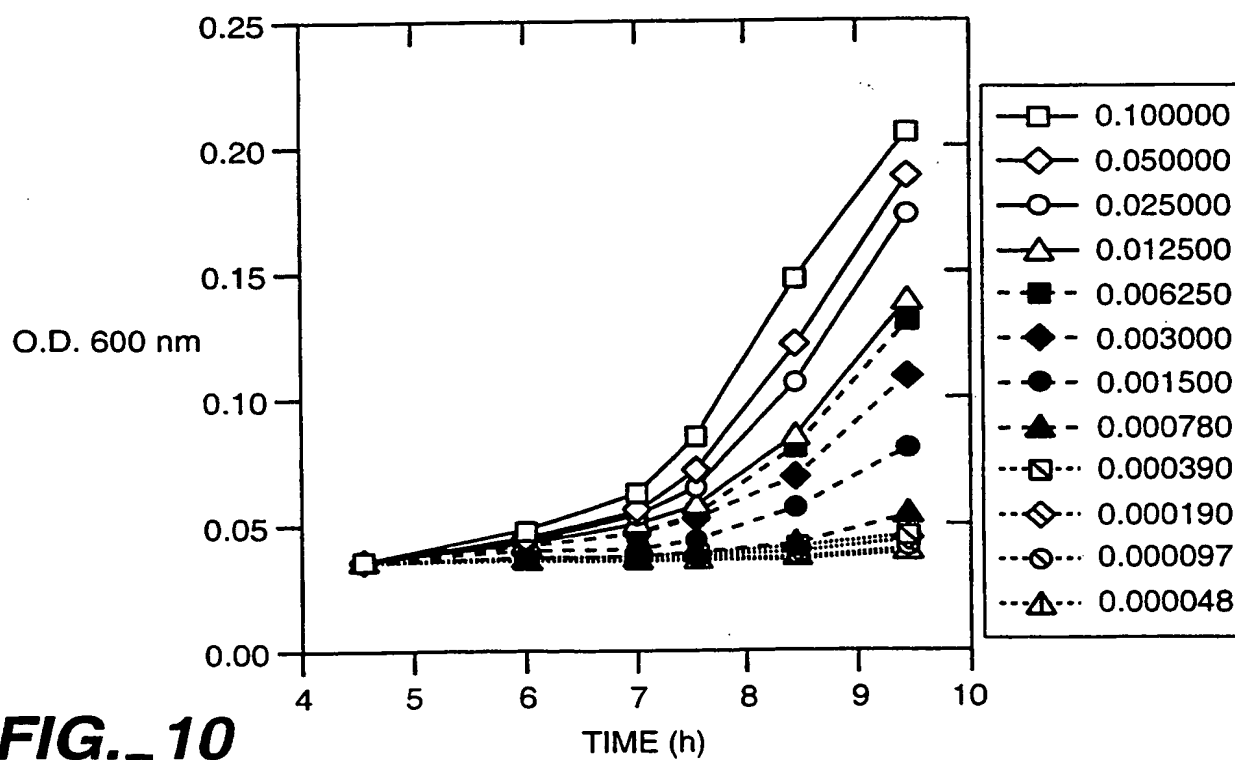
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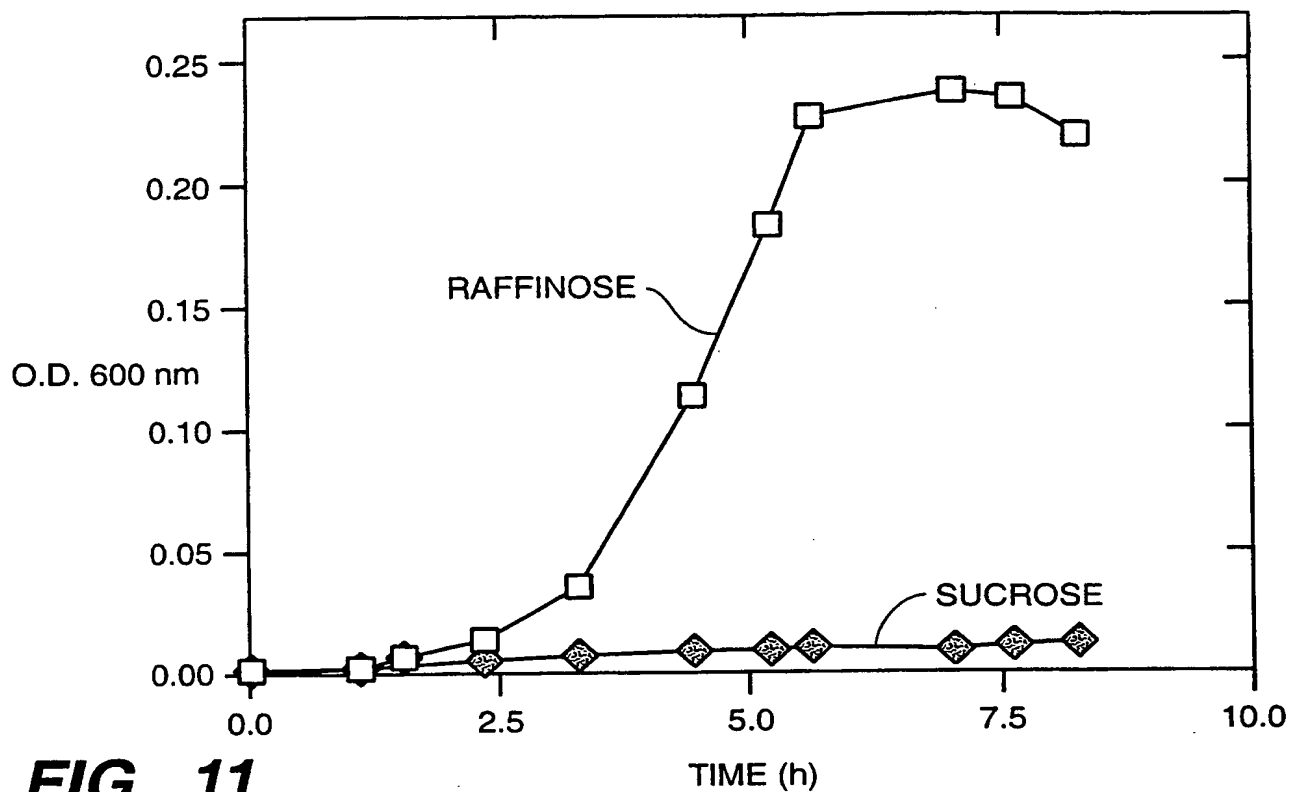
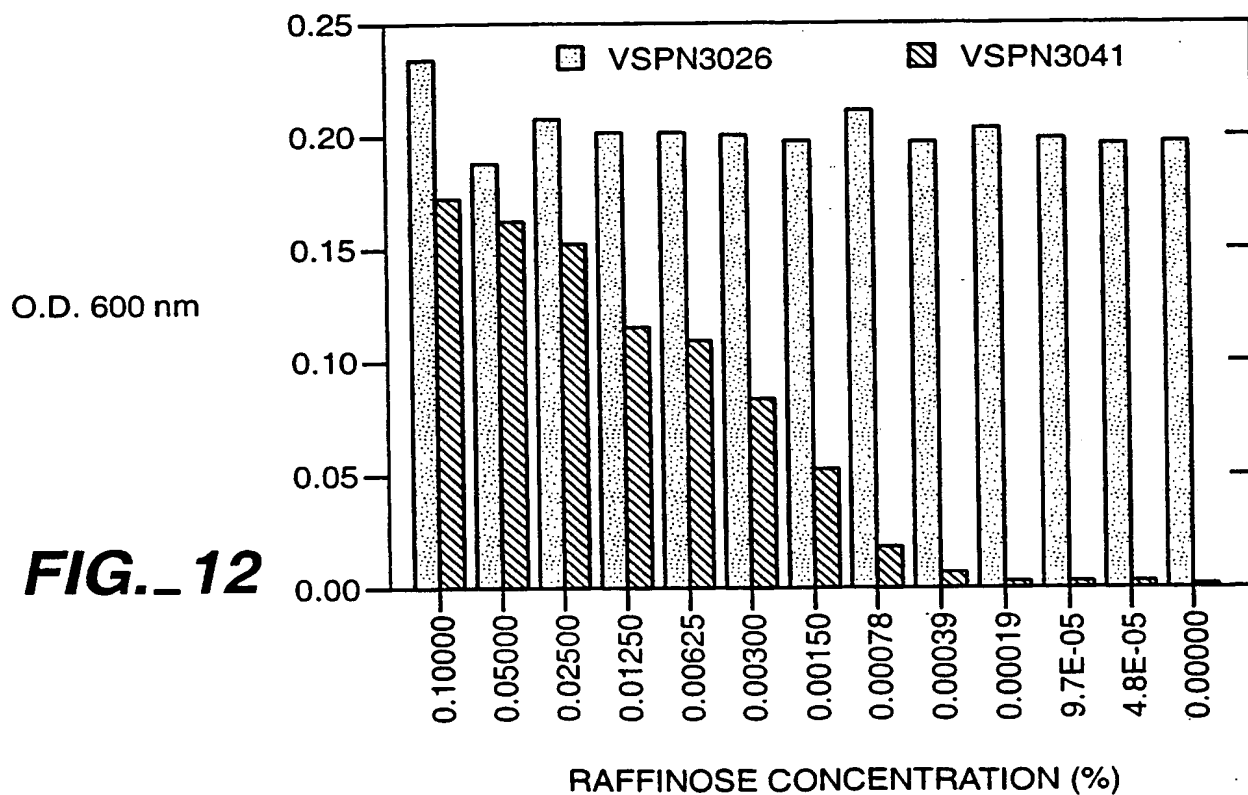
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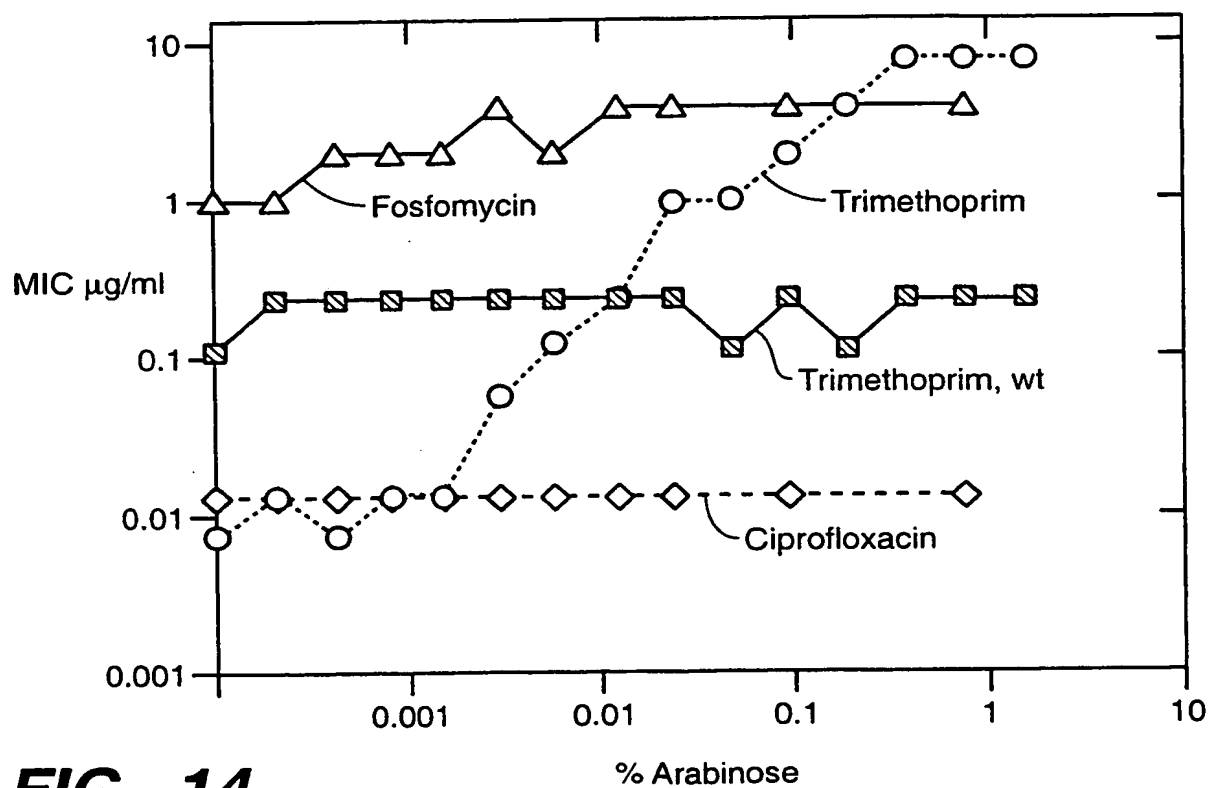
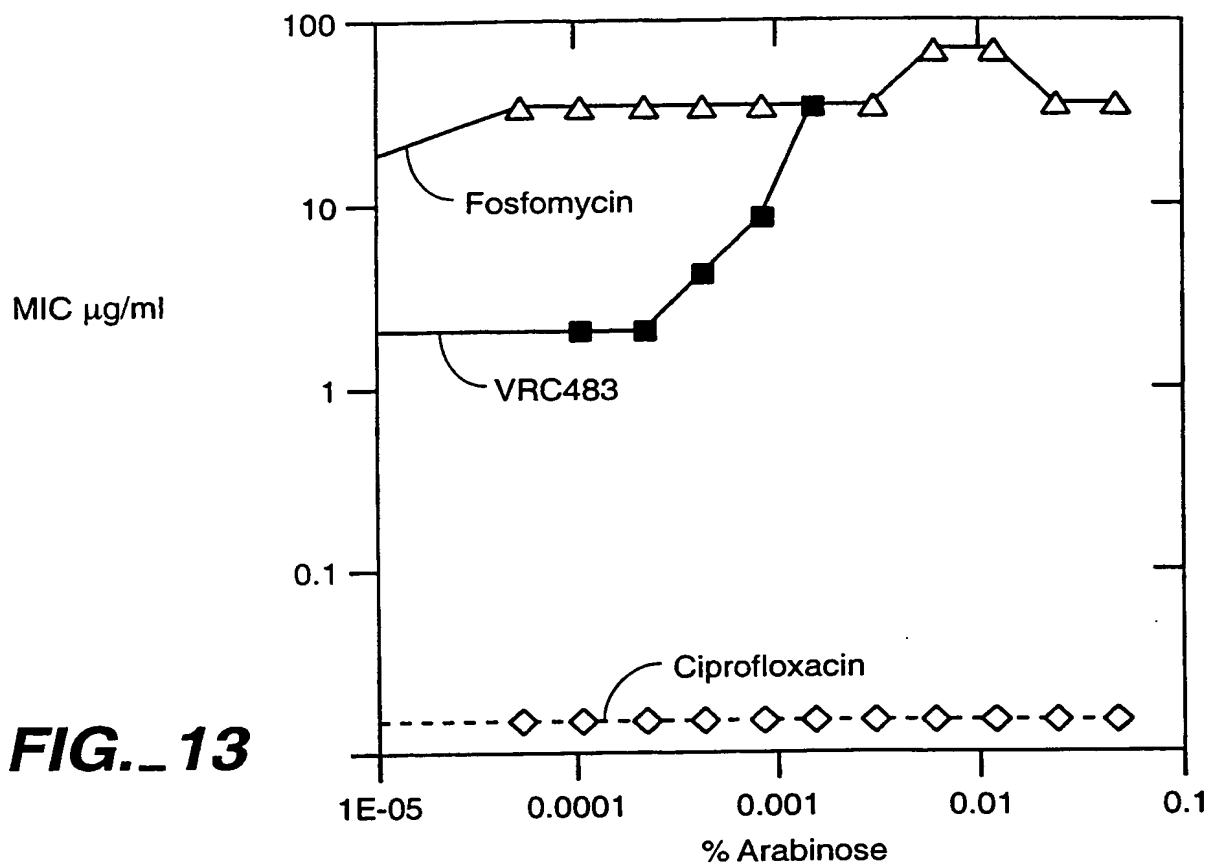
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**FIG. 9****FIG. 10**

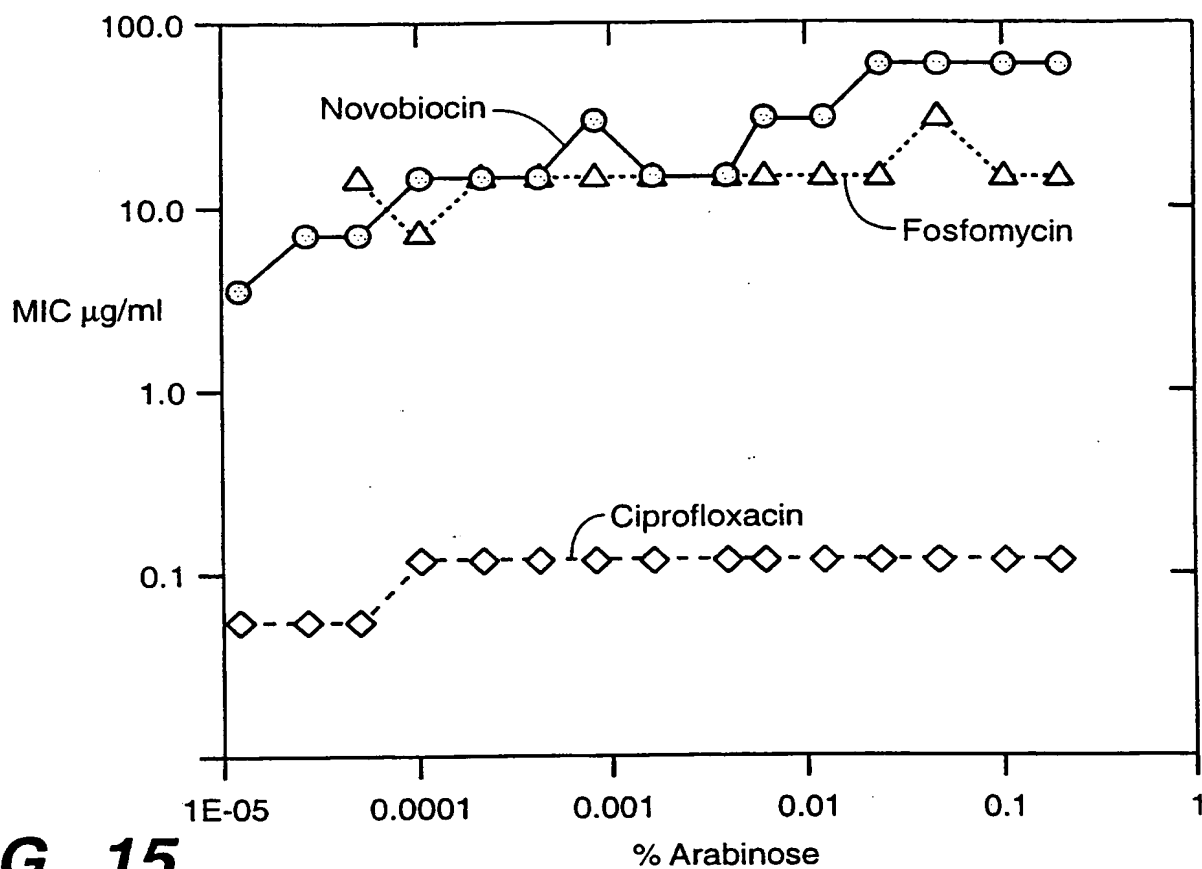
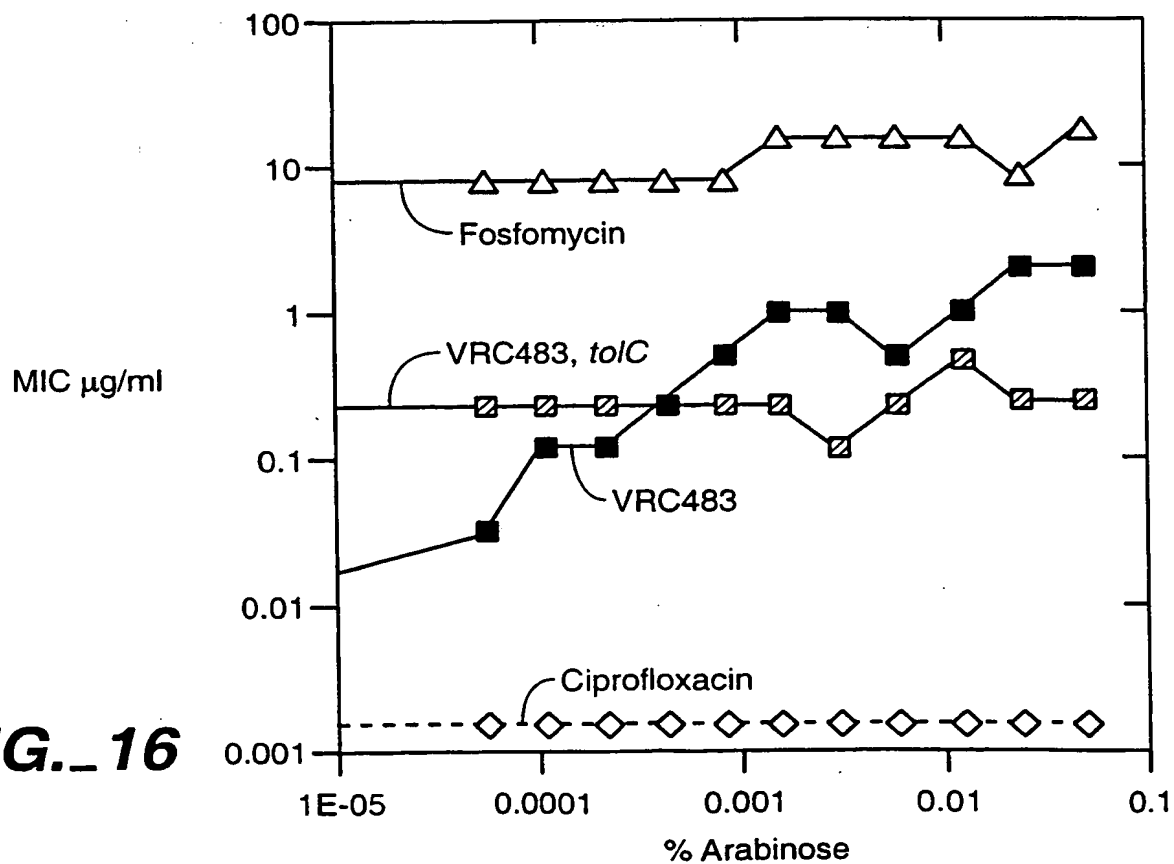
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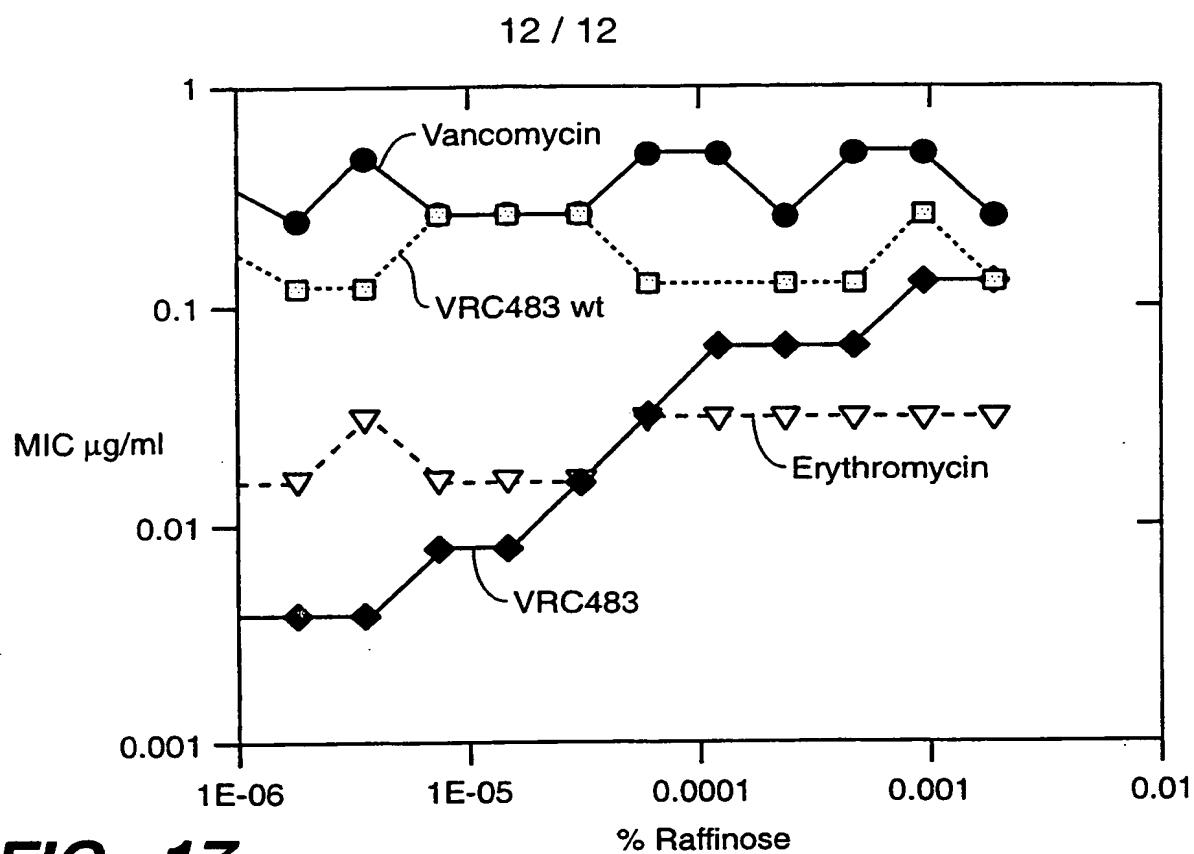
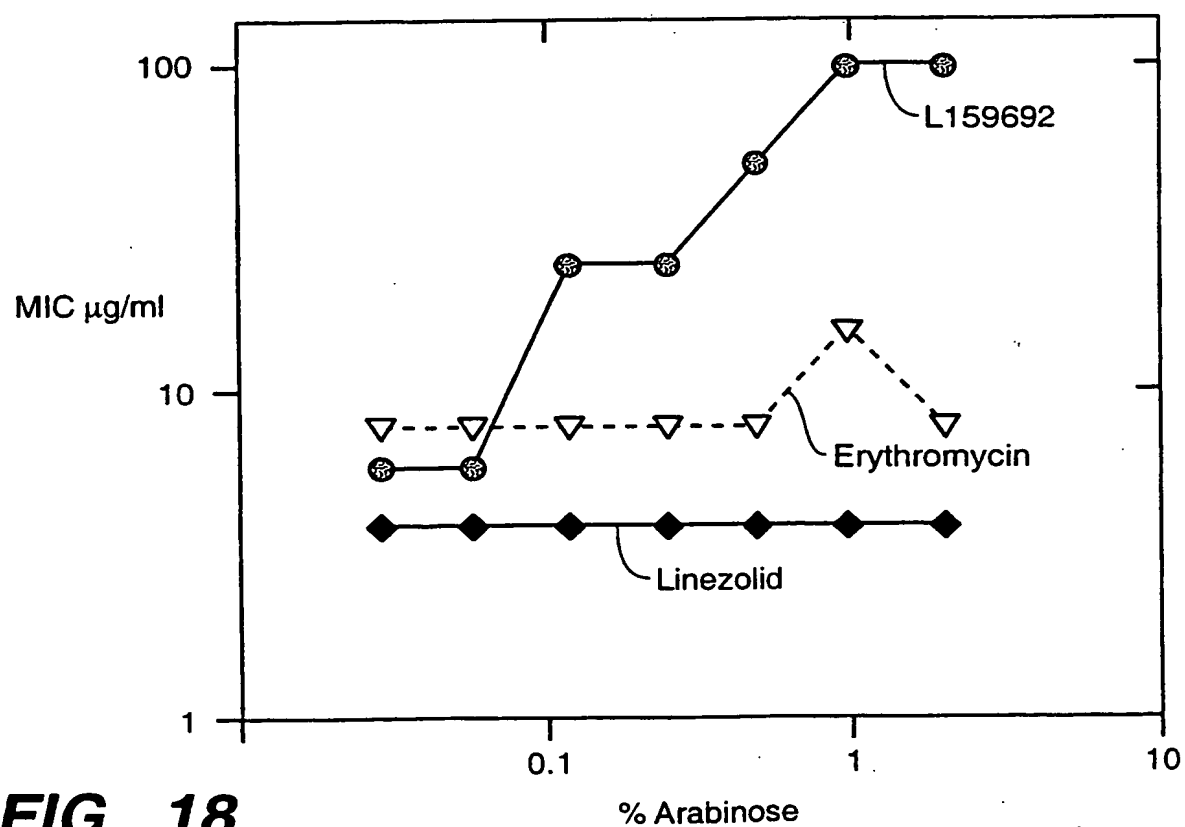
**FIG. 11****FIG. 12**

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**FIG. 15****FIG. 16**

**FIG. 17****FIG. 18**

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INTERNATIONAL SEARCH REPORT

 International application No.
PCT/US99/08164

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : Please See Extra Sheet.

US CL : 435/6, 29, 34, 69.1, 252.1, 253.1, 254.1, 255.1, 320.1, 325

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 29, 34, 69.1, 252.1, 253.1, 254.1, 255.1, 320.1, 325

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GHRAYEB et al. Secretion Cloning Vectors in E. coli. The EMBO Journal. 1984, Vol. 3, No. 10, pages 2437-2442, see entire document.	1-77
A	DEL CASTILLO et al. An Unusual Mechanism for Resistance to the Antibiotic Coumermycin A1. Proc. Natl. Acad. Sci. October 1991, Vol. 88, pages 8860-8864, see the entire document.	1-77
A	CHENG et al. Isolation of Gram Quantities of EcoRI Restriction and Modification Enzymes from an Overproducing Strain. The Journal of Biological Chemistry. 25 September 1984, Vol. 259, No. 18, pages 11571-11575, see the entire document.	1-77

☐ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

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O document referring to an oral disclosure, use, exhibition or other means	
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Date of the actual completion of the international search

03 JULY 1999

Date of mailing of the international search report

24 AUG 1999

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INTERNATIONAL SEARCH REPORT

International application No.

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A. CLASSIFICATION OF SUBJECT MATTER:

IPC (6):

C07H 21/04; C12N 1/12, 1/14, 1/20, 15/63; C12P 21/02; C12Q 1/00, 1/02, 1/04

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, BIOSIS, EMBASE, MEDLINE, DERWENT

search terms: cell? express? basal? polypeptide? protein? ma? dna? untranslat? enzym? transcri? pbad? control?
essential? arabinose? ribose?



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(21) International Application Number: PCT/US99/12251 (22) International Filing Date: 2 June 1999 (02.06.99) (30) Priority Data: 60/087,757 2 June 1998 (02.06.98) US 09/177,953 23 October 1998 (23.10.98) US (71) Applicant (for all designated States except US): ISIS PHARMACEUTICALS, INC. [US/US]; 2292 Faraday Avenue, Carlsbad, CA 92008 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): SANGHVI, Yogesh [US/US]; 2169 Wandering Road, Encinitas, CA 92024 (US). MANOHARAN, Muthiah [US/US]; 7634 Reposado Drive, Carlsbad, CA 92009 (US). RAVIKUMAR, Vasulinga, T. [IN/US]; 6606 Vireo Court, Carlsbad, CA 92009 (US). (74) Agents: CALDWELL, John, W. et al.; Woodcock Washburn Kurtz MacKiewicz & Norris LLP, 46th floor, One Liberty Place, Philadelphia, PA 19103 (US).		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: ACTIVATORS FOR OLIGONUCLEOTIDE SYNTHESIS		
(57) Abstract The present invention relates to improved methods for the preparation of nucleoside phosphoramidites and oligonucleotides. In one aspect, the methods of the invention are used to prepare phosphitylating reagents using pyridinium salts as activators. In a further aspect, the methods of the invention are used to prepare internucleoside linkages using activators which include at least one pyridinium salt and at least one substituted imidazole. In a further aspect, methods are provided for the preparation of internucleoside linkages between nucleosides having 2'-substituents using imidazolium or benzimidazolium salts as an activator. In a further aspect, methods are provided for the preparation of internucleoside linkages between nucleosides having bioreversible protecting group that confers enhanced chemical and biophysical properties, without exocyclic amine protection, using imidazolium or benzimidazolium salts as an activator.		

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ACTIVATORS FOR OLIGONUCLEOTIDE SYNTHESIS

CROSS REFERENCE TO RELATED APPLICATIONS

The present application claims benefit of U.S. application ser. no. 09/177,953, filed October 23, 1998, and
5 U.S. provisional application no. 60/087,757, filed June 2, 1998, the contents of each of which are hereby incorporated by reference in their entirety.

FIELD OF THE INVENTION

The present invention relates to improved methods
10 for the preparation of oligonucleotides and nucleoside phosphoramidites. More particularly, the methods utilize activators that have certain advantages over conventional activators used in the preparation of nucleoside phosphoramidites, and in their coupling to form oligomers.
15 More specific objectives and advantages of the invention will hereinafter be made clear or become apparent to those skilled in the art during the course of explanation of preferred embodiments of the invention.

BACKGROUND OF THE INVENTION

20 The study of oligonucleotides has become a key area of interest for many reasons including potential uses in therapeutic and diagnostic applications (Agrawal, S., TIBTECH, 1996, 14, 375-382; Marr, J., Drug Discovery Today,

- 2 -

1996, 1, 94-102; Rush, W., *Science*, 1997, 276, 1192-1193). One of the more interesting applications of oligonucleotides is the ability to modulate gene and protein function in a sequence specific manner. A direct result of studying
5 oligonucleotides including their analogs in variety of applications is the need for large quantities of compounds having high purity. Presently, the synthesis of oligonucleotides and their analogs remains a tedious and costly process. There remains an ongoing need in this area
10 for developing improved synthetic processes that facilitate the synthesis of oligonucleotides.

Phosphoramidites are important building blocks for the synthesis of oligonucleotides. The most commonly used process in oligonucleotide synthesis using solid phase
15 chemistries is the phosphoramidite approach. In a similar process the support used is a soluble support (Bonora et al., *Nucleic Acids Res.*, 1993, 21, 1213-1217). The phosphoramidite approach is also widely used in solution phase chemistries for oligonucleotide synthesis. Deoxyribo-
20 nucleoside phosphoramidite derivatives (Becaucage et al., *Tetrahedron Lett.*, 1981, 22, 1859-1862) have also been used in the synthesis of oligonucleotides.

Phosphoramidites for a variety of nucleosides are commercially available through a myriad of vendors. 3'-O-
25 phosphoramidites are the most widely used amidites but the synthesis of oligonucleotides can involve the use of 5'-O- and 2'-O- phosphoramidites (Wagner et al., *Nucleosides & Nucleotides*, 1997, 17, 1657-1660; Bhan et al., *Nucleosides & Nucleotides*, 1997, 17, 1195-1199). There are also many
30 phosphoramidites available that are not nucleosides (Cruachem Inc., Dulles, VA; Clontech, Palo Alto, CA).

One of the steps in the phosphoramidite approach to oligonucleotide synthesis is the 3'-O-phosphitylation of 5'-O-protected nucleosides. Additionally, exocyclic amino

- 3 -

groups and other functional groups present on nucleobase moieties are normally protected prior to phosphitylation. Traditionally phosphitylation of nucleosides is performed by treatment of the protected nucleosides with a

5 phosphitylating reagent such as chloro-(2-cyanoethoxy)-N,N-diisopropylaminophosphine which is very reactive and does not require an activator or 2-cyanoethyl-N,N,N',N'-tetraiso-propylphosphorodiamidite (bis amidite reagent) which requires an activator. After preparation the nucleoside 3'-
10 O-phosphoramidite is coupled to a 5'-OH group of a nucleoside, nucleotide, oligonucleoside or oligonucleotide.

The activator most commonly used in phosphitylation reactions is 1H-tetrazole. There are inherent problems with the use of 1H-tetrazole, especially
15 when performing larger scale syntheses. For example, 1H-tetrazole is known to be explosive. According to the material safety data sheet (MSDS) 1H-tetrazole (1H-tetrazole, 98%) can be harmful if inhaled, ingested or absorbed through the skin. The MSDS also states that 1H-
20 tetrazole can explode if heated above its melting temperature of 155°C and may form very sensitive explosive metallic compounds. In addition, 1H-tetrazole is known to Hence 1H-tetrazole requires special handling during its storage, use, and disposal.

25 Aside from its toxicity and explosive nature 1H-tetrazole is acidic and can cause deblocking of the 5'-O-protecting group and can also cause depurination during the phosphitylation step of amidite synthesis (Krotz et al., *Tetrahedron Lett.*, 1997, 38, 3875-3878). Inadvertent
30 deblocking of the 5'-O-protecting group is also a problem when chloro-(2-cyanoethoxy)-N,N-diisopropylaminophosphine is used. Recently, trimethylchlorosilane has been used as an activator in the phosphitylation of 5'-O-DMT nucleosides with bis amidite reagent but this reagent is usually
35 contaminated with HCl which leads to deprotection and

- 4 -

formation of undesired products (Dabkowski, W., et al. *Chem. Comm.*, 1997, 877). The results for this phosphitylation are comparable to those for 1H-tetrazole.

Activators with a higher pKa (i.e., less acidic) than 1H-tetrazole (pKa 4.9) such as 4,5-dicyanoimidazole (pKa 5.2) have been used in the phosphitylation of 5'-O-DMT thymidine (Vargeese, C., *Nucleic Acids Res.*, 1998, 26, 1046-1050).

A variety of activators have been used in the coupling of phosphoramidites in addition to 1H-tetrazole. 5-Ethylthio-1H-tetrazole (Wincott, F., et al., *Nucleic Acids Res.* 1995, 23, 2677) and 5-(4-nitrophenyl)-1H-tetrazole (Pon, R.T., *Tetrahedron Lett.*, 1987, 28, 3643) have been used for the coupling of sterically crowded ribonucleoside monomers e.g. for RNA-synthesis. The pKa's for these activators are 4.28 and 3.7 (1:1 ethanol:water), respectively. The use of pyridine hydrochloride/imidazole (pKa 5.23 (water)) as an activator for coupling of monomers was demonstrated by the synthesis of a dimer (Gryaznov, S.M., Letsinger, L.M., *Nucleic Acids Res.*, 1992, 20, 1879). Benzimidazolium triflate (pKa 4.5 (1:1 ethanol:water)) (Hayakawa et al., *J. Org. Chem.*, 1996, 61, 7996-7997) has been used as an activator for the synthesis of oligonucleotides having bulky or sterically crowded phosphorus protecting groups such as aryloxy groups. The use of imidazolium triflate (pKa 6.9 (water)) was demonstrated for the synthesis of a dimer in solution (Hayakawa, Y.; Kataoka, M., *Nucleic Acids and Related Macromolecules: Synthesis, Structure, Function and Applications*, September 4-9, 1997, Ulm, Germany). The use of 4,5-dicyanoimidazole as an activator for the synthesis of nucleoside phosphoramidite and several 2'-modified oligonucleotides including phosphorothioates has also been reported (Vargeese, *supra.*).

- 5 -

Another disadvantage to using 1H-tetrazole is the cost of the reagent. The 1997 Aldrich Chemical Company catalog lists 1H-tetrazole at over ten dollars a gram for 98% material. The 99+% pure material lists for over forty
5 seven dollars per gram. This reagent is used in excess of the stoichiometric amount of nucleoside present in the reaction mixture resulting in considerable cost especially during large scale syntheses.

The solubility of 1H-tetrazole is also a factor in
10 the large scale synthesis of phosphoramidites, oligonucleotides and their analogs. The solubility of 1H-tetrazole is about 0.5 M in acetonitrile. This low solubility is a limiting factor on the volume of solvent that is necessary to run a phosphitylation reaction. An
15 activator having higher solubility would be preferred to allow the use of minimum volumes of reactions thereby also lowering the cost and the production of waste effluents. Furthermore, commonly used 1H-tetrazole (0.45 M solution) for oligonucleotide synthesis precipitates 1H-tetrazole when
20 the room-temperature drops below 20 °C. Thus, blocking the lines on the automated synthesizer.

Due to ongoing clinical demand (See, for example, Crooke et al., *Biotechnology and Genetic Engineering Reviews*, 1998, 15, 121-157) the synthesis of
25 oligonucleotides and their analogs is being performed utilizing increasingly larger scale reactions than in the past. One of the most common processes used in the synthesis of these compounds utilizes phosphoramidites that are routinely prepared and used in conjunction with an
30 activator. There exists a need for phosphitylation activators that poses less hazards, are less acidic, and less expensive than activating agents that are currently being used, such as 1H-tetrazole. This invention is directed to this, as well as other, important ends.

35

- 6 -

SUMMARY OF THE INVENTION

In one aspect, the present invention presents improved methods for preparing phosphitylated compounds comprising the steps of:

5 providing a compound having a hydroxyl group;
 reacting said compound with a phosphitylating reagent in the presence of a pyridinium salt in a solvent under conditions of time, temperature and pressure effective to yield said phosphitylated compound.

10 In some preferred embodiments of the invention, the compound having a hydroxyl group is a nucleoside, preferably a 5'-protected nucleoside having a 3'-hydroxyl group. In further preferred embodiments, the compound is a nucleoside dimer having a 3' or 5'-hydroxyl group. In still
15 further preferred embodiments, said compound is a nucleoside having a 5' or 2' hydroxyl group.

 In further preferred embodiments, the compound having a free hydroxyl group is an oligonucleotide or oligonucleotide analog having a 3' or 5' hydroxyl group.

20 In some preferred embodiments of the invention, the phosphitylating reagent is bis amidite reagent (2-cyanoethyl-N,N,N',N'-tetraisopropylphosphorodiamidite), bis(N,N-diisopropylamino)-2-methyltrifluoroacetylaminomethoxyphosphine or bis(N,N-diisopropylamino)-2-diphenyl-
25 methylsilylethoxyphosphine.

 In further preferred embodiments of the invention, the pyridinium salt is pyridinium hydrochloride, pyridinium trifluoroacetate or pyridinium dichloroacetate.

 In further preferred embodiments of the invention,
30 the solvent is dichloromethane, acetonitrile, ethyl acetate, tetrahydrofuran or a mixture thereof.

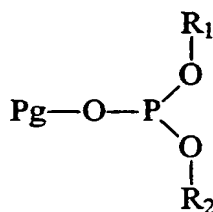
 In further preferred embodiments, the activator is bound to a solid support. In Still further preferred embodiments, the activator is a polyvinyl pyridinium salt.

35 In a further aspect, the present invention

- 7 -

provides improved methods for the preparation of intersugar linkages. In preferred embodiments, the methods of the invention are used in the preparation of oligonucleotides via standard solid phase oligonucleotide regimes.

5 In some preferred embodiments, the present invention presents methods for the preparation of a compound of Formula I:



I

10 wherein:

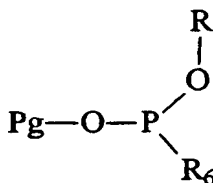
R_1 is a mononucleoside or an oligonucleotide;

R_2 is a nucleoside linked to a solid support, or an oligonucleotide linked to a solid support;

Pg is a phosphorus protecting group;

15 comprising:

providing a phosphoramidite of Formula II:



II

wherein R_6 is $-\text{N}(\text{R}_7)_2$ wherein R_7 is alkyl having from one to
20 about six carbons; or R_7 is a heterocycloalkyl or heterocycloalkenyl ring containing from 4 to 7 atoms, and having up to 3 heteroatoms selected from nitrogen, sulfur, and oxygen;

and reacting said phosphoramidite with a hydroxyl

- 8 -

group of a nucleoside linked to a solid support, or an oligonucleotide linked to a solid support;

said reaction being performed in the presence of an activating reagent, said activating reagent comprising at least one pyridinium salt and at least one substituted imidazole.

Also provided in accordance with the present invention are methods for the preparation of an oligonucleotide comprising the steps of:

10 providing a 3'-mononucleoside phosphoramidite or 3'-oligonucleotide phosphoramidite; and

reacting said 3'-mononucleoside phosphoramidite or 3'-oligonucleotide phosphoramidite with the 5'-hydroxyl of a nucleoside, nucleotide, or oligonucleotide in the presence of an activating reagent;

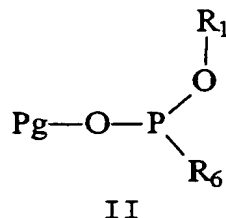
said activating reagent comprising at least one pyridinium salt and at least one substituted imidazole.

In some preferred embodiments, the 3'-mononucleoside phosphoramidite or oligonucleotide phosphoramidite is reacted with the 5'-hydroxyl of a solid-support bound nucleoside, nucleotide or oligonucleotide.

In further preferred embodiments of the foregoing methods of the invention, the oligonucleotide comprises phosphorothioate intersugar linkages.

25 The present invention also provides synthetic methods comprising:

providing a phosphoramidite of Formula II:



30

wherein

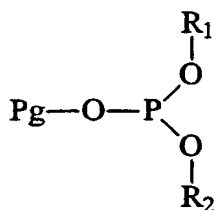
- 9 -

R_1 is a mononucleoside or an oligonucleotide;

Pg is a phosphorus protecting group;

R_2 is $-N(R_7)_2$, wherein R_7 is alkyl having from one to about six carbons; or R_7 is a heterocycloalkyl or heterocycloalkenyl ring containing from 4 to 7 atoms, and having up to 3 heteroatoms selected from nitrogen, sulfur, and oxygen; and

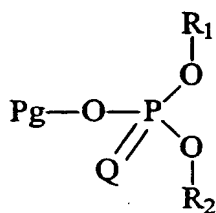
reacting said phosphoramidite with a hydroxyl group of a nucleoside linked to a solid support, or an oligonucleotide linked to a solid support, to form a compound of Formula I:



I

said reaction being performed in the presence of an activating reagent, said activating reagent comprising at least one pyridinium salt and at least one substituted imidazole; and

oxidizing or sulfurizing said compound to form a compound of Formula III:



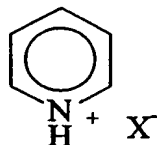
III

wherein Q is O or S, with S being preferred.

In some preferred embodiments of the foregoing methods, the substituted imidazole is 1-methylimidazole.

In further preferred embodiments of the foregoing methods, the pyridinium salt has the formula

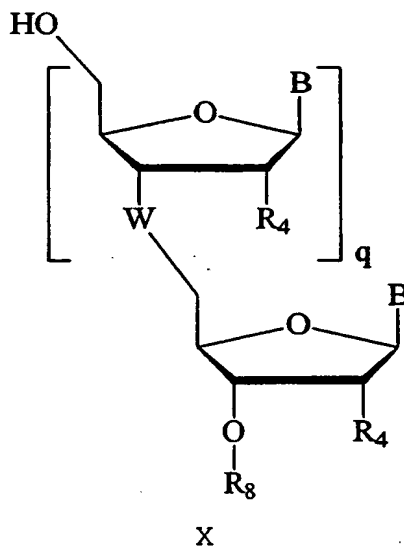
- 10 -



where X^- is trifluoroacetate, ^-O -mesyl, ^-O -tosyl, ^-Br , ^-O -trifluorosulfonyl, hexafluorophosphate or tetrafluoroborate, with trifluoroacetate being preferred.

5 In a further aspect of the invention, synthetic methods are provided comprising:

providing a compound of Formula X:



10 wherein:

B is a nucleobase;

R_8 is H, a hydroxyl protecting group, or a linker connected to a solid support;

W is an optionally protected internucleoside

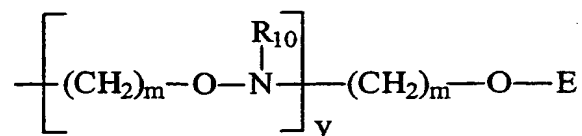
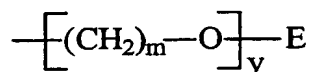
15 linkage;

q is 0 to about 50;

R_4 is H, F, O-R, S-R or N-R(R_{10});

R is H, a protecting group, or has one of the formulas:

- 11 -



where

each m is independently from 1 to 10;

y is from 0 to 10;

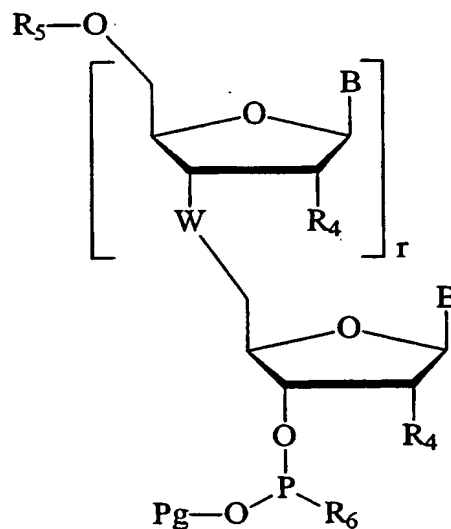
5 E is H, a hydroxyl protecting group, C₁-C₁₀ alkyl, N(R₁₀)(R₁₁) or N=C(R₁₀)(R₁₁); substituted or unsubstituted C₁-C₁₀ alkyl, C₂-C₁₀ alkenyl, C₂-C₁₀ alkynyl, wherein the substitutions are selected from one or several halogen, cyano, carboxy, hydroxy, nitro and mercapto
10 residues;

each R₁₀ or R₁₁ is, independently, H, substituted or unsubstituted C₁-C₁₀ alkyl, C₂-C₁₀ alkenyl, C₂-C₁₀ alkynyl, wherein the substitutions are selected from one or several halogen, cyano, carboxy, hydroxy, nitro and mercapto
15 residues; alkylthioalkyl, a nitrogen protecting group, or R₁₀ and R₁₁, together, are a nitrogen protecting group or wherein R₁₀ and R₁₁ are joined in a ring structure that can include at least one heteroatom selected from N and O;

or R is -CH₂-CH₂-O-CH₂-CH₂-N(R₁₀)(R₁₁);

20 reacting the compound of Formula X in the presence of an activating reagent with a compound of Formula XI:

- 12 -



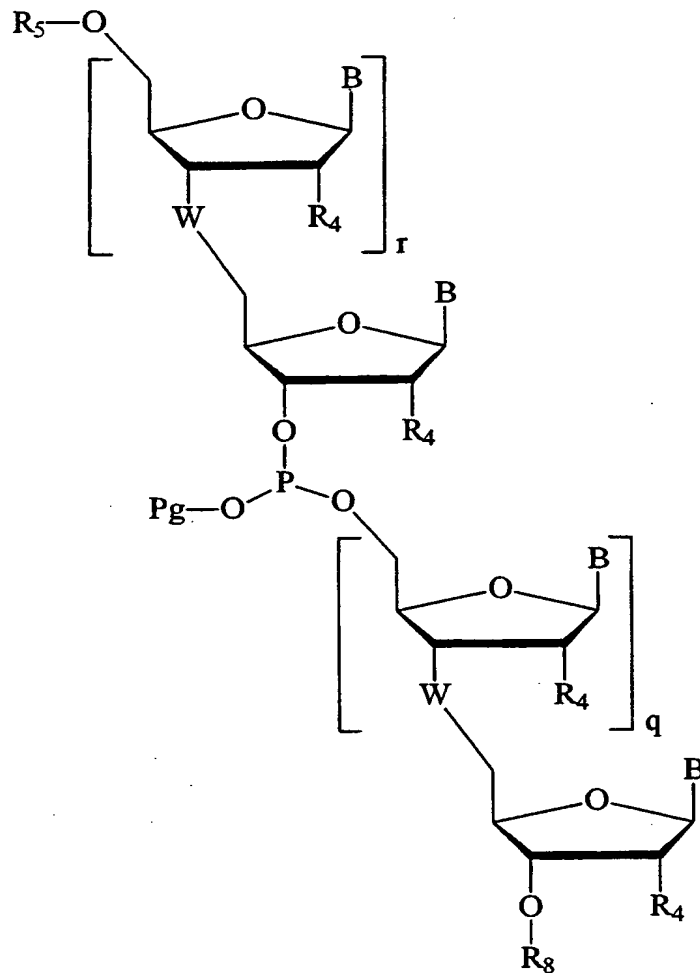
XI

where r is 0 to about 50;

5 R_5 is a hydroxyl protecting group;

R_6 is $-N(R_7)_2$ wherein R_7 is alkyl having from one to about six carbons; or R_7 is a heterocycloalkyl or heterocycloalkenyl ring containing from 4 to 7 atoms, and having up to 3 heteroatoms selected from nitrogen, sulfur,
 10 and oxygen;
 to form a compound of Formula XII:

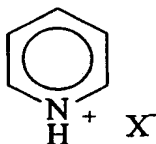
- 13 -



XII

wherein said activating reagent comprises at least one pyridinium salt and one substituted imidazole.

5 In some preferred embodiments, the pyridinium salt has the formula:



where X^- is trifluoroacetate, ^-O -mesyl, ^-O -tosyl, ^-Br , ^-O -trifluorosulfonyl, hexafluorophosphate, or
 10 tetrafluoroborate, with trifluoroacetate being preferred.

In further preferred embodiments, the substituted

- 14 -

imidazole is 1-methylimidazole.

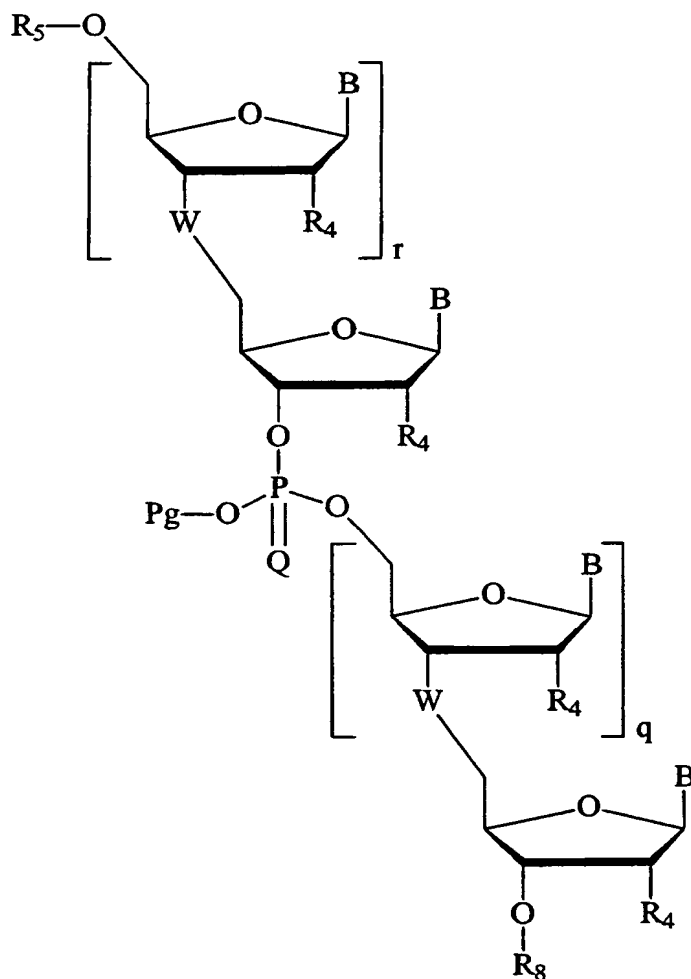
In some preferred embodiments, R_8 is a linker connected to a solid support.

In further preferred embodiments, R_4 is $-O-R$
5 wherein R has the formula $-[-(CH_2)_m-O-]_y-E$; m is 2, y is 1;
and E is CH_3 , $-N(R_{10})(R_{11})$, or $-CH_2-CH_2-N(R_{10})(R_{11})$.

In further preferred embodiments, r is 0. In
still further preferred embodiments, R_6 is diisopropylamino.

Preferably, P_g is $-CH_2CH_2CN$, $-CH_2CH=CHCH_2CN$, para-
10 $CH_2C_6H_4CH_2CN$, $-(CH_2)_{2-5}N(H)COCF_3$, $-CH_2CH_2Si(C_6H_5)_2CH_3$, or
 $-CH_2CH_2N(CH_3)COCF_3$, with $-CH_2CH_2CN$ being more preferred.

Some preferred embodiment of the methods further
comprising oxidizing or sulfurizing the compound of Formula
XII to form a compound of Formula XIII:



XIII

where Q is O or S, with S being preferred.

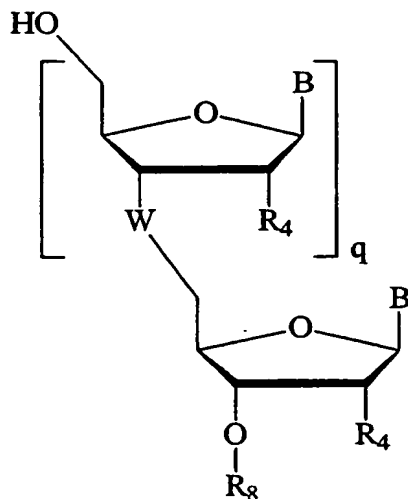
Some further preferred embodiments of the methods further comprising a capping step, which is preferably performed prior to oxidation.

Some further preferred embodiments further comprising the step of cleaving the oligomeric compound to produce a further compound of formula X.

In a further aspect of the invention, methods are provided for the preparation of internucleoside linkages between nucleosides having 2'-substituents, using an activating reagent that is preferably an imidazolium triflate. In some preferred embodiments, these methods comprise:

- 16 -

providing a compound of Formula X:



X

5 wherein:

B is a nucleobase;

R₈ is H, a hydroxyl protecting group, or a linker connected to a solid support;

W is an optionally protected internucleoside

10 linkage;

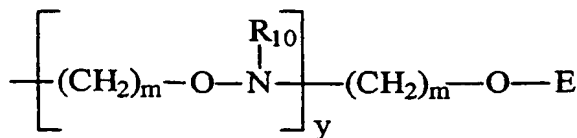
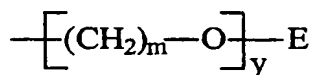
q is 0 to about 50;

R₄ is H, F, O-R, S-R or N-R(R₁₀);

R is H, a protecting group, or has one of the

formulas:

15



where

each m is independently from 1 to 10;

y is from 0 to 10;

E is H, a hydroxyl protecting group, C₁-C₁₀

- 17 -

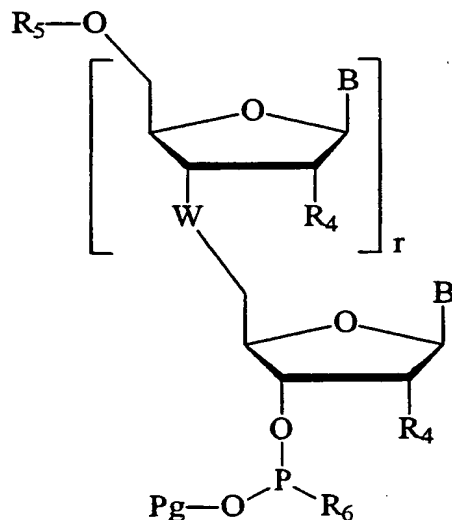
alkyl, $N(R_{10})(R_{11})$ or $N=C(R_{10})(R_{11})$; substituted or unsubstituted C_1-C_{10} alkyl, C_2-C_{10} alkenyl, C_2-C_{10} alkynyl, wherein the substitutions are selected from one or several halogen, cyano, carboxy, hydroxy, nitro and mercapto
 5 residues; and

each R_{10} or R_{11} is, independently, H, substituted or unsubstituted C_1-C_{10} alkyl, C_2-C_{10} alkenyl, C_2-C_{10} alkynyl, wherein the substitutions are selected from one or several halogen, cyano, carboxy, hydroxy, nitro and mercapto
 10 residues; alkylthioalkyl, a nitrogen protecting group, or R_{10} and R_{11} , together, are a nitrogen protecting group or wherein R_{10} and R_{11} are joined in a ring structure that can include at least one heteroatom selected from N and O;

or R is $-CH_2-CH_2-O-CH_2-CH_2-N(R_{10})(R_{11})$;

15 provided that R_{14} is not H or OH;

reacting the compound of Formula X in the presence of an activator with a compound of Formula XI:



XI

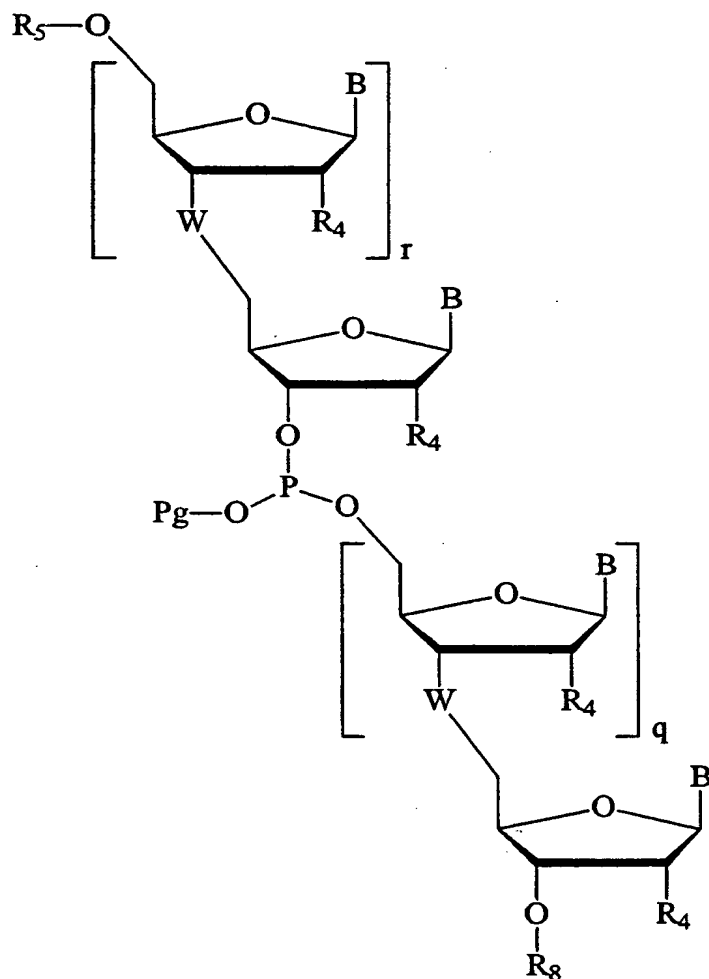
where r is 0 to about 50;

R_5 is a hydroxyl protecting group;

R_6 is $-N(R_7)_2$ wherein R_7 is alkyl having from one to about six carbons; or R_7 is a heterocycloalkyl or

heterocycloalkenyl ring containing from 4 to 7 atoms, and having up to 3 heteroatoms selected from nitrogen, sulfur, and oxygen;

to form a compound of Formula XII:



XII

wherein the activator has the formula G^+U^- ,

where

G^+ is selected from the group consisting of pyridinium, imidazolium, and benzimidazolium; and

U^- is selected from the group consisting of hexafluorophosphate, tetrafluoroborate, triflate, hydrochloride, trifluoroacetate, dichloroacetate, ^-O -mesyl, ^-O -tosyl, ^-Br , and ^-O -trifluorosulfonyl.

- 19 -

Preferably, the activator is imidazolium triflate.

In some preferred embodiments, R_8 is a linker connected to a solid support. In further preferred embodiments, R_4 is $-O-R$ wherein R has the formula $-[-$

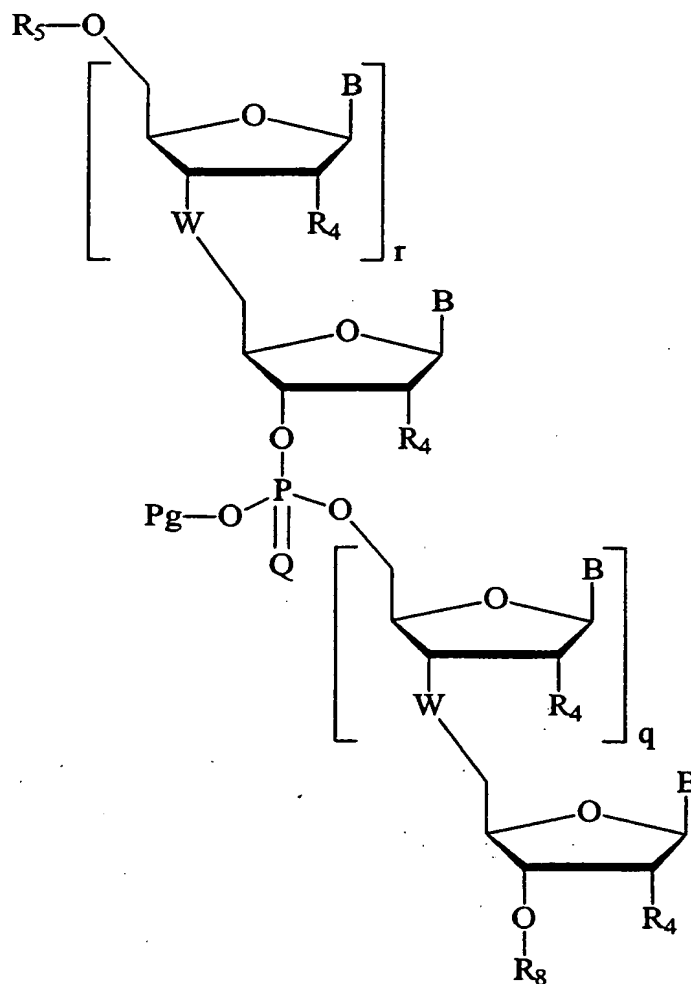
5 $(CH_2)_m-O-]_y-E$; m is 2, y is 1; and E is CH_3 , $-N(R_{10})(R_{11})$, or $-CH_2-CH_2-N(R_{10})(R_{11})$.

In further preferred embodiments, r is 0. In still further preferred embodiments, R_6 is diisopropylamino.

Preferably, P_g is $-CH_2CH_2CN$, $-CH_2CH=CHCH_2CN$, para-
10 $CH_2C_6H_4CH_2CN$, $-(CH_2)_{2-5}N(H)COCF_3$, $-CH_2CH_2Si(C_6H_5)_2CH_3$, or $-CH_2CH_2N(CH_3)COCF_3$, with $-CH_2CH_2CN$ being more preferred.

Some further preferred embodiments further comprise oxidizing or sulfurizing the compound of Formula XII to form a compound of Formula XIII:

- 20 -



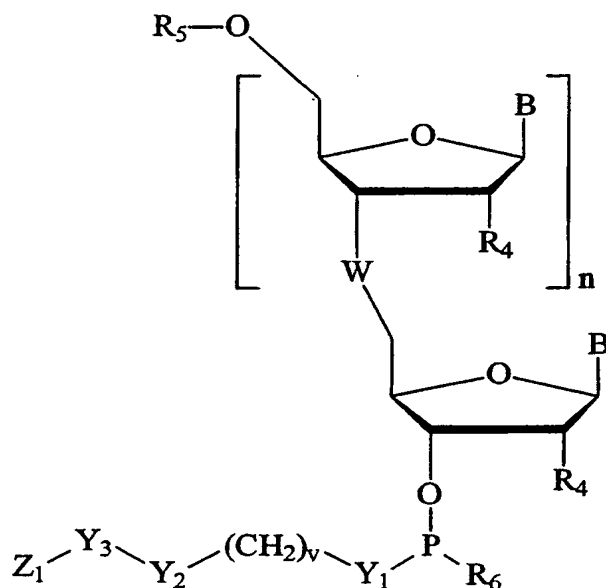
where Q is O or S, with S being preferred.

Some further preferred embodiments of the methods
 5 further comprising a capping step, which is preferably
 performed prior to oxidation.

Some further preferred embodiments further
 comprising the step of cleaving the oligomeric compound to
 produce a further compound of formula X.

10 In a further aspect of the invention, synthetic
 methods are provided comprising:
 providing a compound of Formula XX:

- 21 -



XX

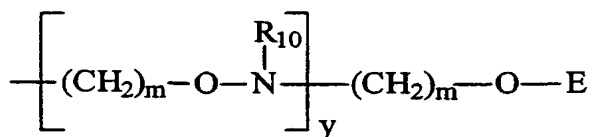
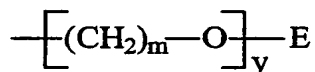
wherein:

 R_4 is H, F, O-R, S-R or N-R(R_{10});

5

R is H, a protecting group, or has one of the

formulas:



where

each m is independently from 1 to 10;

10

y is from 0 to 10;

E is H, a hydroxyl protecting group, $\text{C}_1\text{--C}_{10}$ alkyl, $\text{N}(\text{R}_{10})$ (R_{11}) or $\text{N}=\text{C}(\text{R}_{10})$ (R_{11}); substituted orunsubstituted $\text{C}_1\text{--C}_{10}$ alkyl, $\text{C}_2\text{--C}_{10}$ alkenyl, $\text{C}_2\text{--C}_{10}$ alkynyl,

wherein the substitutions are selected from one or several

15 halogen, cyano, carboxy, hydroxy, nitro and mercapto

residues; and

each R_{10} or R_{11} is, independently, H, substituted or

- 22 -

unsubstituted C₁-C₁₀ alkyl, C₂-C₁₀ alkenyl, C₂-C₁₀ alkynyl, wherein the substitutions are selected from one or several halogen, cyano, carboxy, hydroxy, nitro and mercapto residues; alkylthioalkyl, a nitrogen protecting group, or R₁₀ and R₁₁, together, are a nitrogen protecting group or wherein R₁₀ and R₁₁ are joined in a ring structure that can include at least one heteroatom selected from N and O;

or R is -CH₂-CH₂-O-CH₂-CH₂-N(R₁₀)(R₁₁);

R₅ is a hydroxyl protecting group;

Z₁ is aryl having 6 to about 14 carbon atoms or alkyl having from one to about six carbon atoms;

Y₁ is O or S;

Y₂ is O or S;

Y₃ is C(=O) or S;

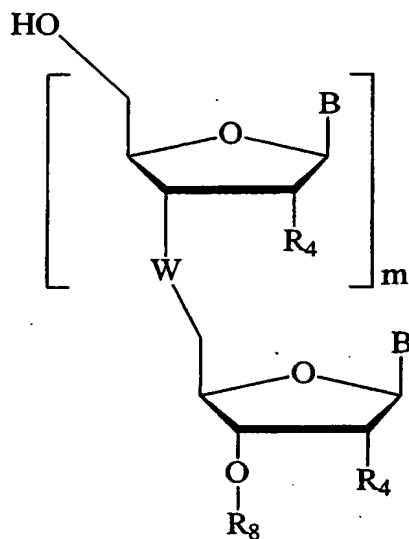
v is 2 to about 4;

B is a nucleobase;

R₆ is -N(R₇)₂ wherein R₇ is alkyl having from one to about six carbons; or R₇ is a heterocycloalkyl or heterocycloalkenyl ring containing from 4 to 7 atoms, and having up to 3 heteroatoms selected from nitrogen, sulfur, and oxygen;

reacting said compound of Formula XX with a compound of Formula XXI:

- 23 -

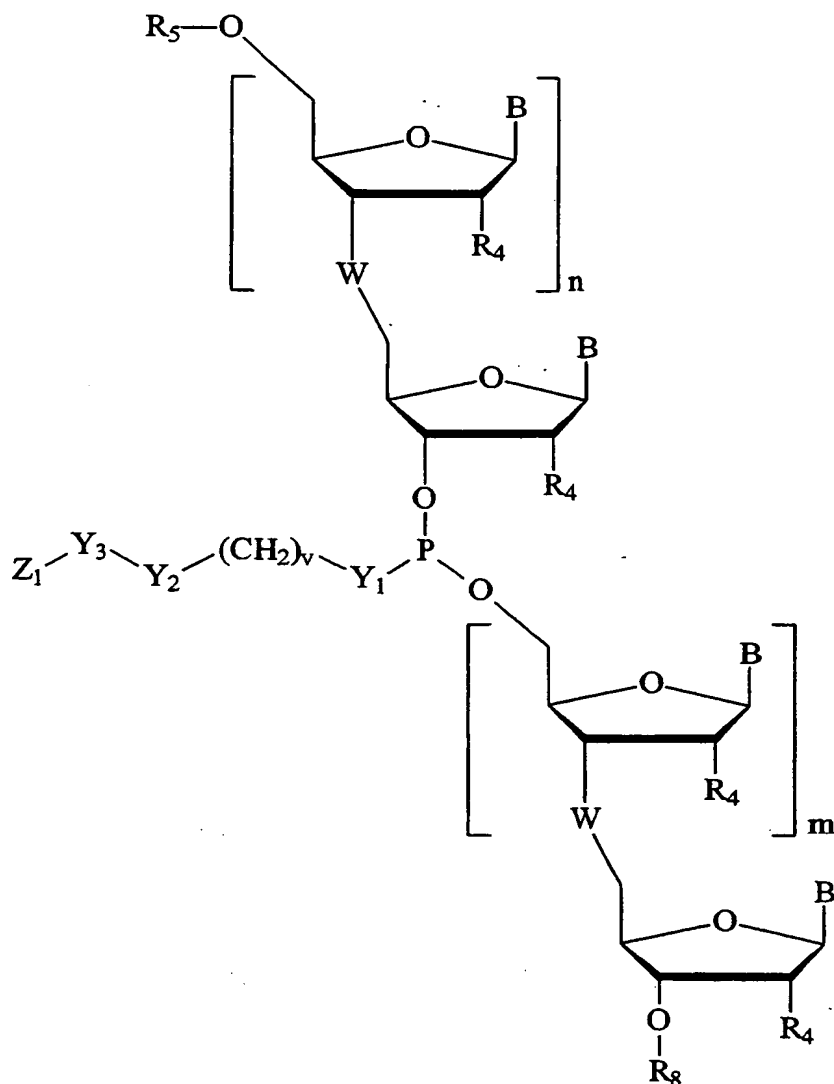


XXI

wherein:

- R₈ is H, a hydroxyl protecting group, or a linker
5 connected to a solid support;
in the presence of an activator to form a compound
of Formula XXII:

- 24 -



XXII

wherein the activator has the formula G^+U^- ,

where

- 5 G^+ is selected from the group consisting of pyridinium, imidazolium, and benzimidazolium; and
- U^- is selected from the group consisting of hexafluorophosphate, tetrafluoroborate, triflate, hydrochloride, trifluoroacetate, dichloroacetate, ^-O -mesyl,
- 10 ^-O -tosyl, ^-Br , and ^-O -trifluorosulfonyl;
- or said activator is a substituted imidazolium triflate.
- Preferably, the activator is imidazolium triflate.

- 25 -

In some preferred embodiments, v is 2; and Y_3 is $C(=O)$. In further preferred embodiments, Z is methyl, phenyl or t -butyl, with t -butyl being preferred.

In some preferred embodiments, n is 0. In further
5 preferred embodiments, R_8 is a linker to a solid support.

In some preferred embodiments, Y_1 and Y_2 are each O . In other preferred embodiments, Y_1 and Y_2 are each S . In still further preferred embodiments, Y_1 is O and Y_2 is S .

Preferably, each R_7 is isopropyl.

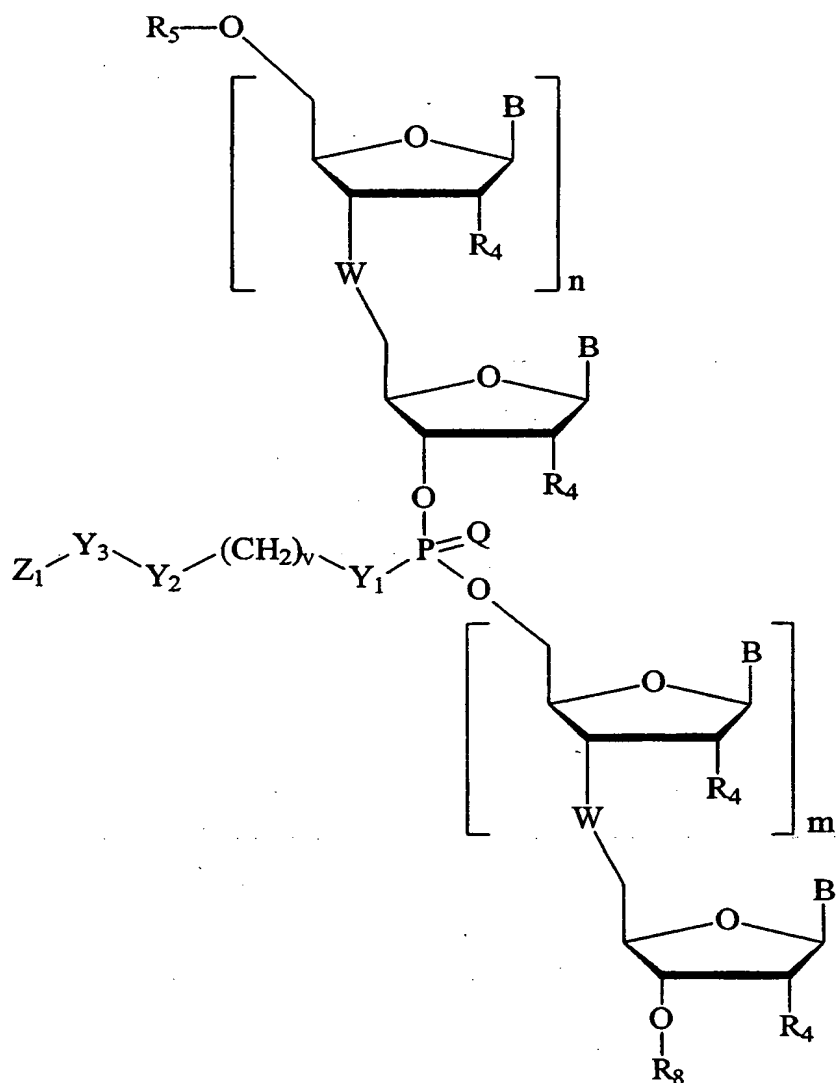
10 In some preferred embodiments, n is 0; R_3 is H , R_6 is diisopropylamino; Y_1 is O ; Y_2 is S ; and Z is methyl or t -butyl, with t -butyl being preferred.

In some preferred embodiments of each of the foregoing methods, each constituent nucleobase "B" is devoid
15 of exocyclic amine protection.

Preferably, W is an optionally protected phosphodiester, phosphorothioate, phosphorodithioate, or alkyl phosphonate internucleotide linkage.

Some preferred embodiments of the foregoing
20 methods further comprise oxidizing or sulfurizing the compounds of Formula XXII to form a compound of Formula XXIII:

- 26 -



XXIII

where Q is O or S.

Some further preferred embodiments of the methods further comprising a capping step, which is preferably performed prior to oxidation.

Some further preferred embodiments further comprising the step of cleaving the oligomeric compound to produce a further compound of formula XXI.

In some preferred embodiments, G⁺ is pyridinium and U⁻ is hexafluorophosphate or tetrafluoroborate, with hexafluoroborate being preferred.

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In further preferred embodiments, G^+ is imidazolium or benzimidazolium and U^- is selected from the group consisting of triflate, hydrochloride, trifluoroacetate, dichloroacetate, ^-O -mesyl, ^-O -tosyl, ^-Br ,
5 and ^-O -trifluorosulfonyl.

In other preferred embodiments, G^+ is imidazolium or benzimidazolium and U^- is selected from the group consisting of hexafluorophosphate, tetrafluoroborate, and triflate.

10 In further preferred embodiments, G^+ is imidazolium or benzimidazolium and U^- is selected from the group consisting of hydrochloride, trifluoroacetate, dichloroacetate, ^-O -mesyl, ^-O -tosyl, ^-Br , and ^-O -trifluorosulfonyl.

15 In still further preferred embodiments, G^+ is imidazolium and U^- is selected from the group consisting of hexafluorophosphate, tetrafluoroborate, triflate, hydrochloride, trifluoroacetate, dichloroacetate, ^-O -mesyl, ^-O -tosyl, ^-Br , and ^-O -trifluorosulfonyl.

20 In still further preferred embodiments, U^- is selected from the group consisting of hexafluorophosphate, tetrafluoroborate, and triflate.

In further preferred embodiments, U^- is selected from the group consisting of hydrochloride,
25 trifluoroacetate, dichloroacetate, ^-O -mesyl, ^-O -tosyl, ^-Br , and ^-O -trifluorosulfonyl.

In further preferred embodiments, G^+ is benzimidazolium and U^- is selected from the group consisting of hexafluorophosphate, tetrafluoroborate, triflate,
30 hydrochloride, trifluoroacetate, dichloroacetate, ^-O -mesyl, ^-O -tosyl, ^-Br , and ^-O -trifluorosulfonyl.

In further preferred embodiments, G^+ is benzimidazolium and U^- is hexafluorophosphate, tetrafluoroborate, or triflate.

35 In further preferred embodiments, G^+ is

- 28 -

benzimidazolium and U⁻ is selected from the group consisting of hydrochloride, trifluoroacetate, dichloroacetate, ⁻O-mesyl, ⁻O-tosyl, ⁻Br, and ⁻O-trifluorosulfonyl.

In some preferred embodiments, the activator is substituted or unsubstituted imidazolium triflate, with
5 unsubstituted imidazolium triflate being preferred.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a scheme showing intermediates and products in a series of phosphitylation reactions according
10 to the invention.

Figure 2 is a list of activating reagents suitable for use in the present invention.

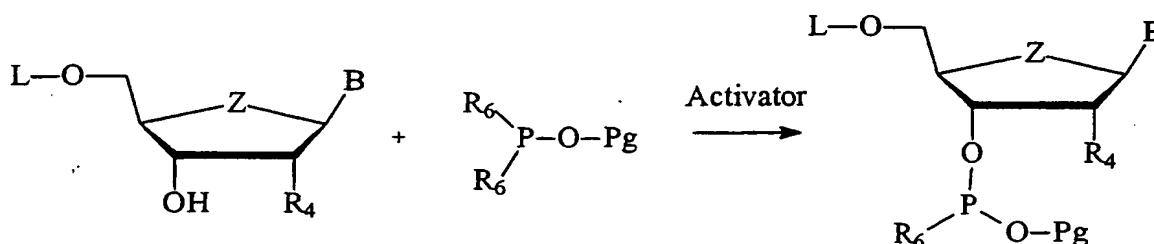
Figure 3 is a list of activating reagents suitable for use in the present invention.

15 Figure 4 is an electropherogram comparing the efficiency of tetrazole activator and pyridinium trifluoroacetate / 1-methylimidazole activator.

DESCRIPTION OF PREFERRED EMBODIMENTS

The present invention describes improved methods
20 for, *inter alia*, phosphitylating compounds having a free ("unblocked") hydroxyl group. In some preferred embodiments, the compound to be phosphitylated is a mononucleoside, an oligonucleotide, or analog thereof.

A large number of compounds are amenable to the
25 improved process of the present invention. A general scheme utilizing some preferred starting materials is illustrated below:



wherein

L is a hydroxyl protecting group, a nucleotide, a nucleoside, an oligonucleotide or and oligonucleoside;

5 Z is O, S, CH₂ or NR₁₀;

B is a nucleobase or a modified nucleobase;

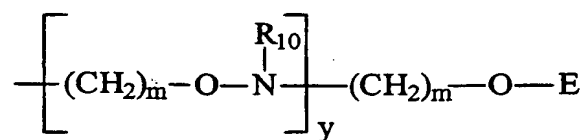
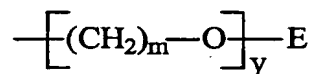
Pg is a phosphorus protecting group that is preferably -CH₂CH₂CN,

-CH₂CH=CHCH₂CN, para-CH₂C₆H₄CH₂CN, -(CH₂)₂₋₅N(H)COCF₃,

10 -CH₂CH₂Si(C₆H₅)₂CH₃, or -CH₂CH₂N(CH₃)COCF₃;

R₄ is H, F, O-R, S-R or N-R(R₁₀);

R is H, a protecting group, or has one of the formulas:



15 where

each m is independently from 1 to 10;

y is from 0 to 10;

E is H, a hydroxyl protecting group, C₁-C₁₀ alkyl, N(R₁₀)(R₁₁) or N=C(R₁₀)(R₁₁); substituted or

20 unsubstituted C₁-C₁₀ alkyl, C₂-C₁₀ alkenyl, C₂-C₁₀ alkynyl,

wherein the substitutions are selected from one or several halogen, cyano, carboxy, hydroxy, nitro and mercapto residues; and

- 30 -

each R_{10} or R_{11} is, independently, H, substituted or unsubstituted C_1 - C_{10} alkyl, C_2 - C_{10} alkenyl, C_2 - C_{10} alkynyl, wherein the substitutions are selected from one or several halogen, cyano, carboxy, hydroxy, nitro and mercapto residues; alkylthioalkyl, a nitrogen protecting group, or R_{10} and R_{11} , together, are a nitrogen protecting group or wherein R_{10} and R_{11} are joined in a ring structure that can include at least one heteroatom selected from N and O; or R is $-CH_2-CH_2-O-CH_2-CH_2-N(R_{10})(R_{11})$.

10 The initial step in the phosphitylation scheme illustrated above is the activation of the phosphorus atom of the phosphitylating reagent via protonation. The activator donates a proton to the phosphorus atom of the phosphitylating reagent (i.e., a P^{III} compound having at
15 least one phosphorus/oxygen bond) thereby activating the reagent. The activation involves formation of a salt with the corresponding anion of the activator. When the phosphitylating reagent is activated the phosphorus atom undergoes nucleophilic attack by a free hydroxyl group
20 displacing a diisopropylamino group which forms a salt with the anion of the activator. As depicted above the free hydroxyl group is a 3' hydroxyl group but the attacking nucleophile could alternatively be a 5' hydroxyl group (Wagner, T., and Pfeleiderer, W., *Nucleosides & Nucleotides*,
25 1997, 16, 1657-1660) or a 2' hydroxyl group (Bhan et al., *Nucleosides & Nucleotides*, 1997, 16, 1195-1199). The nucleophilic attack results in the formation of a stable phosphoramidite ($P^{(III)}$) compound.

In addition to phosphitylation of 3' hydroxyl
30 positions of nucleosides or larger oligomeric structures the present invention is also amenable to phosphitylation of 5', 2', and 1' hydroxyl positions. The present process is also amenable to compounds other than nucleosides. All that is required is that the compound have an unblocked hydroxyl
35 group and be inert to the reaction conditions of

- 31 -

phosphitylation, or, for example, be rendered inert to the reaction conditions by addition of appropriate protecting groups if necessary. There are numerous examples in the literature of phosphitylation of non-nucleosidic compounds
5 such as for example: alkyl groups (Filippov et al., *Nucleosides & Nucleotides*, 1997, 16, 1403-1406); cyclohexoses (Schlienger et al., *Nucleosides & Nucleotides*, 1997, 16, 1325-1329); peptide nucleic acid (Vinayak et al., *Nucleosides & Nucleotides*, 1997, 16, 1653-1656); macrocyclic
10 ligands (Wagner et al., *Nucleosides & Nucleotides*, 1997, 17, 1789-1792), European Patent Application no. EP 0 816 368 A1, entitled "Chemical Phosphorylation of Oligonucleotides and Reactants used therefor, filed July 2, 1997, published January 7, 1998.

15 In preferred embodiments, the methods of the present invention use pyridinium salts as activators during the synthesis of phosphoramidites. Thus, the methods of the invention possess significant advantages over conventional phosphitylation processes. For example, the activators of
20 the present invention can be generated *in situ* by mixing equal molar amounts of the base pyridine and an acid such as for example HCl, CF₃COOH, CHCl₂COOH or CF₃SO₃H (trifluoromethylsulfonic acid). The *in situ* preparation of activators is quick, easy and provides significant benefit in the
25 performance synthesis of phosphoramidites on a large scale. Particularly, at the oligonucleotide manufacturing site, where pyridine and CHCl₂COOH both are used as synthesis reagents, are also useful for the preparation of activator.

The mechanism of phosphoramidite activation has
30 been studied (Vargeese, *supra*; Dahl et al., *Nucleic Acids Research*, 1987, 15, 1729-1743). The first step is the protonation of the trivalent phosphorus. The next step which is slower is the displacement of N,N-diisopropylamine by an activator such as 1-H tetrazole. Typically, in the

- 32 -

phosphitylation step of amidite based oligonucleotide synthesis, the 1-H tetrazole first participates as an acid, and then as a nucleophile.

Although not wanting to be bound by theory it is
5 thought that the mechanism of phosphitylation observed for the preferred pyridinium salts of the invention are not the same as when 1-H tetrazole is employed. As illustrated in the series of experiments of Example 12, the first step is seen as protonation of the phosphitylating reagent via
10 proton transfer from the activator. The second step is seen as the reaction of the activated phosphitylating species directly with the 3'-hydroxyl group of the nucleoside. Hence, the pyridine is not seen as a nucleophilic participant in the reaction scheme.

15 Pyridinium salts are non-explosive which make them substantially easier to store, use and dispose of relative to conventional activators such as 1H-tetrazole. Pyridinium salts and the starting materials necessary to generate them *in situ.*, are safely stored in large quantities. The
20 removal of pyridinium ion from reaction mixtures is easily performed by conversion to pyridine which is easily removed by evaporation. Furthermore, the cost of pyridinium salt is only \$0.10/gram compared to \$47.00/gram for 1H-tetrazole. This cost differential results in substantive cost-savings
25 for large scale manufacture of oligonucleotide drugs.

Another advantage that pyridinium salts have over conventional activators is their solubility in organic solvents. The solubility of pyridinium salts is significantly higher in solvents such as acetonitrile,
30 dichloromethane, and ethyl acetate than 1H-tetrazole. For example the of pyridinium trifluoroacetate in acetonitrile is greater than 1 molar which is more than twice the solubility of 1H-tetrazole in acetonitrile which is about 0.5 molar under identical conditions. As a result of this increased
35 solubility the volume of solvents used during

- 33 -

phosphitylation can be greatly reduced. Another result of the increased solubility is that other solvent systems can be used giving enhanced results that are not feasible with activators such as 1H-tetrazole. The improved process of
5 the present invention is performed using a solvent that can dissolve protected nucleosides. Preferred solvents include dichloromethane, dichloroethane, acetonitrile, tetrahydrofuran, ethyl acetate and mixtures thereof. In a preferred embodiment the improved process is performed using
10 dichloromethane.

The use of pyridinium salts as activators improves the purity of the final phosphitylated material relative to conventional activators such as 1H-tetrazole. This improved purity results from a less acidic reaction medium when
15 pyridinium salts are used. Pyridinium salts also provide a less acidic reaction environment than is observed when using more reactive phosphitylating reagents such as chloro-(2-cyanoethoxy)-N,N-diisopropylaminophosphine. This reduction in acidity leads to no loss of 5'-O-protection (see Example
20 9) which is always a problem with conventional more acidic activators. There is also no depurination seen (see Example 11) with the use of pyridinium activators. The fact that there are less undesired products as a result of depurination and deprotection simplifies purification of
25 desired phosphoramidites.

A number of chemical functional groups present in the nucleosidic compounds of the invention can be protected and subsequently deblocked to the deprotected form. In general, a blocking group renders a chemical functionality
30 of a molecule inert to specific reaction conditions and can later be removed from such functionality in a molecule without substantially damaging or altering the remainder of the molecule (Green and Wuts, Protective Groups in Organic Synthesis, 2d edition, John Wiley & Sons, New York, 1991).
35 Common protecting groups that are routinely used during

oligonucleotide synthesis are disclosed in Agrawal, et al.,
Protocols for Oligonucleotide Conjugates, Eds, Humana Press;
New Jersey, 1994; Vol. 26 pp. 1-72.

Nucleosidic compounds according to the present
5 invention include monomeric and linked nucleosides. The
term "nucleoside" is intended to include naturally occurring
nucleosides and nucleosides having modified nucleobases
and/or modified sugar moieties. Internucleoside linkages
between linked nucleosides comprise native phosphodiester
10 linkages as well as modified linkages such as phosphoro-
thioate linkages. Other internucleoside linkages as is
known in the art are also amenable to the present invention.

As used in the present application the term
"nucleobase" is intended to include naturally occurring
15 nucleobases such as for example adenine, guanine, cytosine,
uridine, and thymine, as well as nucleobases that are
modified such as xanthine, hypoxanthine, 2-aminoadenine, 6-
methyl and other alkyl derivatives of adenine and guanine,
2-propyl and other alkyl derivatives of adenine and guanine,
20 5-halo uracil and cytosine, 6-aza uracil, cytosine and
thymine, 5-uracil (pseudo uracil), 4-thio uracil, 8-halo,
amino, thiol, thioalkyl, hydroxyl and other 8-substituted
adenines and guanines, 5-trifluoromethyl and other 5-
substituted uracils and cytosines, 7-methylguanine. Further
25 purines and pyrimidines include those disclosed in United
States Patent No. 3,687,808, those disclosed in the Concise
Encyclopedia Of Polymer Science And Engineering, pages 858-
859, Kroschwitz, J.I., ed. John Wiley & Sons, 1990, and
those disclosed by Englisch et al., *Angewandte Chemie*,
30 *International Edition* 1991, 30, 613, Limbach, A., et al.,
Nucleic Acids Research, 1994, 22, 2183-2196.

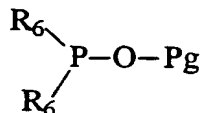
Sugar modifications are known in the prior art and
include for example 2' substituents such as F and 2'-O-
substituents such as substituted or unsubstituted C₁-C₁₀
35 alkyl, C₂-C₁₀ alkenyl, C₂-C₁₀ alkynyl, ethers and polyethers

- 35 -

wherein the substitutions are selected from one or several halogen, cyano, carboxy, hydroxy, nitro and mercapto residues.

Modified internucleoside linkages are known in the prior art and include for example methylphosphonates, monothiophosphates, dithiophosphates, phosphoramidates, phosphate esters, bridged phosphoroamidates, bridged phosphorothioates, bridged methylenephosphonates, dephospho internucleotide analogs with siloxane bridges, carbonate bridges, carboxymethyl ester bridges, acetamide bridges, carbamate bridges, thioether, sulfoxy, sulfono bridges, various "plastic" DNAs, α -anomeric bridges, and borane derivatives.

Phosphitylating reagents that are amenable to the present invention require an activating agent prior to being susceptible to nucleophilic attack from an unprotected 2', 3' or 5' hydroxyl group. Included in this group are phosphitylating reagents having the formula below:



wherein the constituent variables are as defined above.

A more preferred group of phosphitylating reagents includes bis amidite reagent, bis(N,N-diisopropylamino)-2-methyltrifluoroacetylaminooethoxyphosphine and bis(N,N-diisopropylamino)-2-diphenylmethylsilylethoxyphosphine, and bis(N,N-diisopropylamino)-2-(2'-acetoxyloxy)phenylethoxyphosphine.

In a preferred embodiment of the present invention pyridinium salts used as activators are selected to have pK_a 's of from about 5.2 to about 5.9. Preferred pyridinium salts in this group include pyridine hydrochloride,

- 36 -

pyridinium trifluoroacetate and pyridinium dichloroacetate.

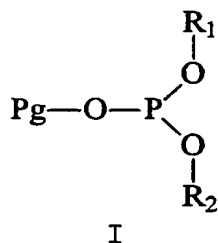
A summary of some phosphitylation activators, their optimal ratio, and exemplary choice of solvent is presented in Table 3 below. Several activators were studied
5 based on their pKa properties, steric bulk/size, cost, safety and scalability during manufacture of phosphoramidites. Also, the activator must act as an acid and have the capacity to transfer a proton to the phosphitylating reagent in an efficient manner. In
10 addition, when phosphitylating 5'-O-DMT-nucleosides the activator should be sufficiently mild to not cause destruction of the acid labile DMT protecting. Activators with pKa between 4.5 - 7 (see Figure 2) were chosen and studied herein. One preferred activator is the pyrimidine
15 derivative 2-amino-4,6-dimethylpyrimidine trifluoroacetate.

Some activators reported in the literature, for example 1-*H* tetrazole and diisopropylammonium tetrazolide, were judged unsuitable due to their high cost, safety in handling, explosive nature, and poor solubility in solvent
20 of choice. Preferred are those derived from pyridinium salts (pyridine hydrochloride, pyridinium trifluoroacetate, pyridinium triflate and pyridinium dichloroacetate) with a common pKa of 5.2. In further preferred embodiments, pyridinium hydrochloride and pyridinium triflate have been
25 shown to be particularly amenable to the methods of the invention, despite the hygroscopic nature of these salts. In particularly preferred embodiments, pyridinium trifluoroacetate activators are employed in the methods of the invention, because they possess an excellent safety
30 profile, low cost, and greater solubility in a range of solvents. Furthermore, the activator pyridinium trifluoroacetate was used to phosphitylate a variety of nucleoside derivatives (1-4, Figure 1) to provide excellent yields. In further preferred embodiments, pyridinium
35 dichloroacetate (see Example 8) also is useful as an

- 37 -

activator in the methods of the invention, and may have an advantage over pyridinium trifluoroacetate because pyridine and dichloroacetic acid is also used as a deblocking solution during oligonucleotide manufacturing, thus avoiding
 5 the storage and handling of an addition reagent.

In a further aspect, the present invention provides novel methods for the preparation of covalent intersugar linkages. In some preferred embodiments, the current invention presents methods for the preparation of a
 10 compound of Formula I:



wherein:

- R_1 is a nucleoside or an oligonucleotide;
- 15 R_2 is a nucleoside linked to a solid support, or an oligonucleotide linked to a solid support;
- Pg is a phosphorus protecting group;
- comprising:
- providing a phosphoramidite of Formula II:



- wherein R_6 is $-\text{N}(\text{R}_7)_2$ wherein R_7 is alkyl having from one to about six carbons; or R_7 is a heterocycloalkyl or heterocycloalkenyl ring containing from 4 to 7 atoms, and
 25 having up to 3 heteroatoms selected from nitrogen, sulfur, and oxygen;

- 38 -

and reacting said phosphoramidite with a hydroxyl group of a nucleoside linked to a solid support, or an oligonucleotide linked to a solid support;

5 said reaction being performed in the presence of an activating reagent, said activating reagent comprising at least one pyridinium salt and at least one substituted imidazole.

The methods of the invention are applicable to the preparation of intersugar linkages including those
10 represented by Formula I above. According to some preferred embodiments of the methods of the invention, a protected phosphoramidite having Formula II is reacted with a hydroxyl group of a sugar moiety of a nucleoside or oligonucleotide. In more preferred embodiments, the nucleoside or
15 oligonucleotide are linked to a solid support, as in, for example, standard solid phase oligonucleotide synthetic regimes.

In the methods of the invention, the reaction of the phosphoramidite and the hydroxyl group is performed in
20 the presence of an activating reagent. As used herein, the term "activating reagent" is intended to mean a reagent that, at a minimum, includes at least one pyridinium salt. It is preferred that the activating reagent also contain at least one imidazole or substituted imidazole, in addition to
25 the pyridinium salt.

The reaction of the phosphoramidite and the hydroxyl group in the presence of the activating reagent can be performed in a solvent, such as acetonitrile.

Also provided in accordance with the present
30 invention are methods for the preparation of an oligonucleotide comprising the steps of:

providing a 3'-mononucleoside phosphoramidite or 3'-oligonucleotide phosphoramidite; and

reacting said 3'-mononucleoside phosphoramidite or
35 3'-oligonucleotide phosphoramidite with the 5'-hydroxyl of a

- 39 -

nucleoside, nucleotide, or oligonucleotide in the presence of an activating reagent;

said activating reagent comprising at least one pyridinium salt and at least one substituted imidazole.

5 In some preferred embodiments, the 3'-mononucleoside phosphoramidite or oligonucleotide phosphoramidite is reacted with the 5'-hydroxyl of a solid-support bound nucleoside, nucleotide or oligonucleotide.

10 In further preferred embodiments of the methods of the invention, the oligonucleotide comprises phosphorothioate intersugar linkages.

The present invention also provides synthetic methods comprising:

providing a phosphoramidite of formula:

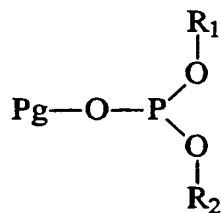


wherein:

R_6 is morpholino or dialkylamino;

Pg is a phosphorus protecting group;

20 and reacting said phosphoramidite with a hydroxyl group of a nucleoside linked to a solid support, or an oligonucleotide linked to a solid support, to form a compound of formula:



wherein:

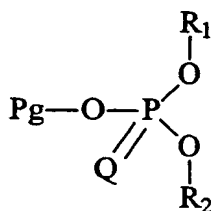
25 R_1 is a mononucleoside or an oligonucleotide;

- 40 -

R_2 is a nucleoside linked to a solid support, or an oligonucleotide linked to a solid support;

said reaction being performed in the presence of an activating reagent, said activating reagent comprising at least one pyridinium salt and at least one substituted imidazole; and

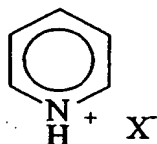
oxidizing or sulfurizing said compound to form a compound of formula:



wherein Q is O or S.

In some preferred embodiments of the forgoing methods, the substituted imidazole is 1-methylimidazole.

In further preferred embodiments, the pyridinium salt has the formula



where X^- is an anion such as, for example, trifluoroacetate, ^-O -mesyl, ^-O -tosyl, ^-Br , ^-O -trifluorosulfonyl, hexafluorophosphate, or tetrafluoroborate, with trifluoroacetate being preferred.

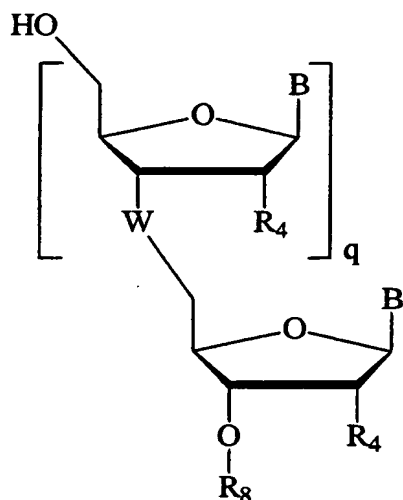
In some preferred embodiments, the compound is a single nucleoside or a nucleoside that is part of a larger molecule such as an oligonucleotide or an oligonucleotide analog. The improved process of the present invention offers significant advantages over traditionally used processes.

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In a further aspect of the invention, synthetic methods are provided comprising:

providing a compound of Formula X:

5



X

wherein:

B is a nucleobase;

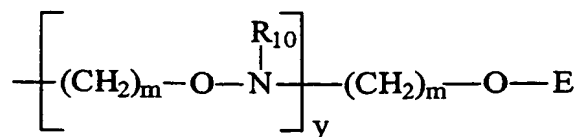
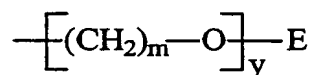
10 R_8 is H, a hydroxyl protecting group, or a linker connected to a solid support;

W is an optionally protected internucleoside linkage;

q is 0 to about 50;

R_4 is H, F, O-R, S-R or N-R(R_{10});

15 R is H, a protecting group, or has one of the formulas:



where

each m is independently from 1 to 10;

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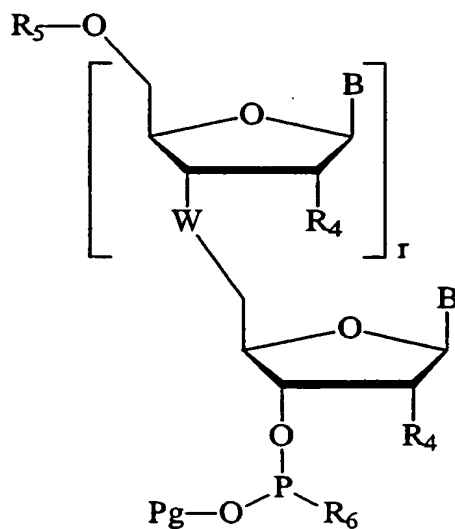
y is from 0 to 10;

E is H, a hydroxyl protecting group, C₁-C₁₀ alkyl, N(R₁₀)(R₁₁) or N=C(R₁₀)(R₁₁); substituted or unsubstituted C₁-C₁₀ alkyl, C₂-C₁₀ alkenyl, C₂-C₁₀ alkynyl, wherein the substitutions are selected from one or several halogen, cyano, carboxy, hydroxy, nitro and mercapto residues; and

each R₁₀ or R₁₁ is, independently, H, substituted or unsubstituted C₁-C₁₀ alkyl, C₂-C₁₀ alkenyl, C₂-C₁₀ alkynyl, wherein the substitutions are selected from one or several halogen, cyano, carboxy, hydroxy, nitro and mercapto residues; alkylthioalkyl, a nitrogen protecting group, or R₁₀ and R₁₁, together, are a nitrogen protecting group or wherein R₁₀ and R₁₁ are joined in a ring structure that can include at least one heteroatom selected from N and O;

or R is -CH₂-CH₂-O-CH₂-CH₂-N(R₁₀)(R₁₁);

reacting the compound of Formula X in the presence of an activating reagent with a compound of Formula XI:



XI

where r is 0 to about 50;

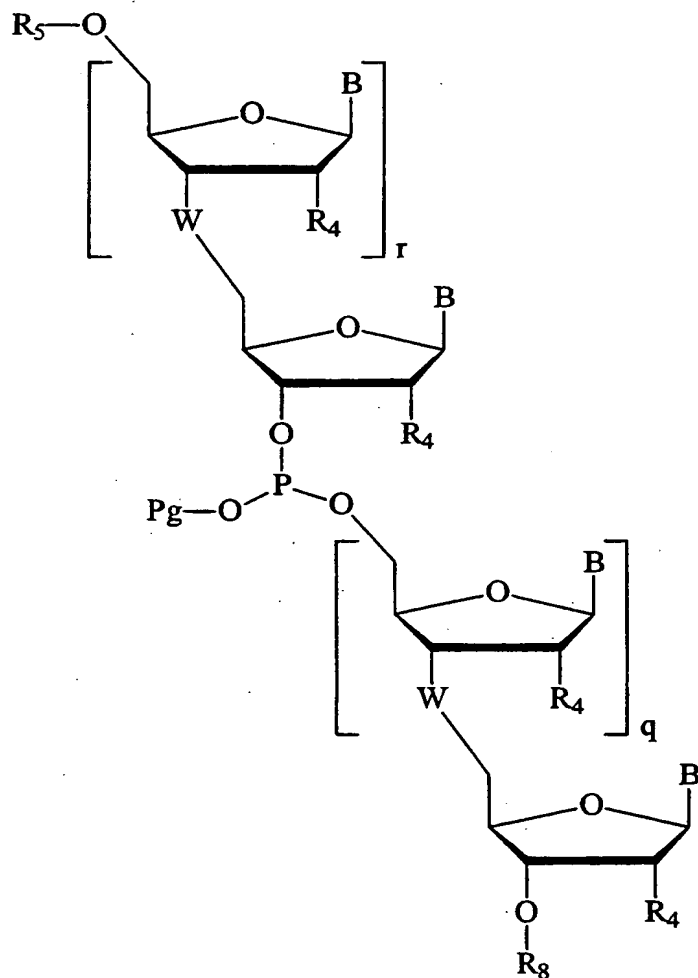
R₅ is a hydroxyl protecting group;

R₆ is -N(R₇)₂ wherein R₇ is alkyl having from one to

- 43 -

about six carbons; or R_7 is a heterocycloalkyl or heterocycloalkenyl ring containing from 4 to 7 atoms, and having up to 3 heteroatoms selected from nitrogen, sulfur, and oxygen;

5 to form a compound of Formula XII:



XII

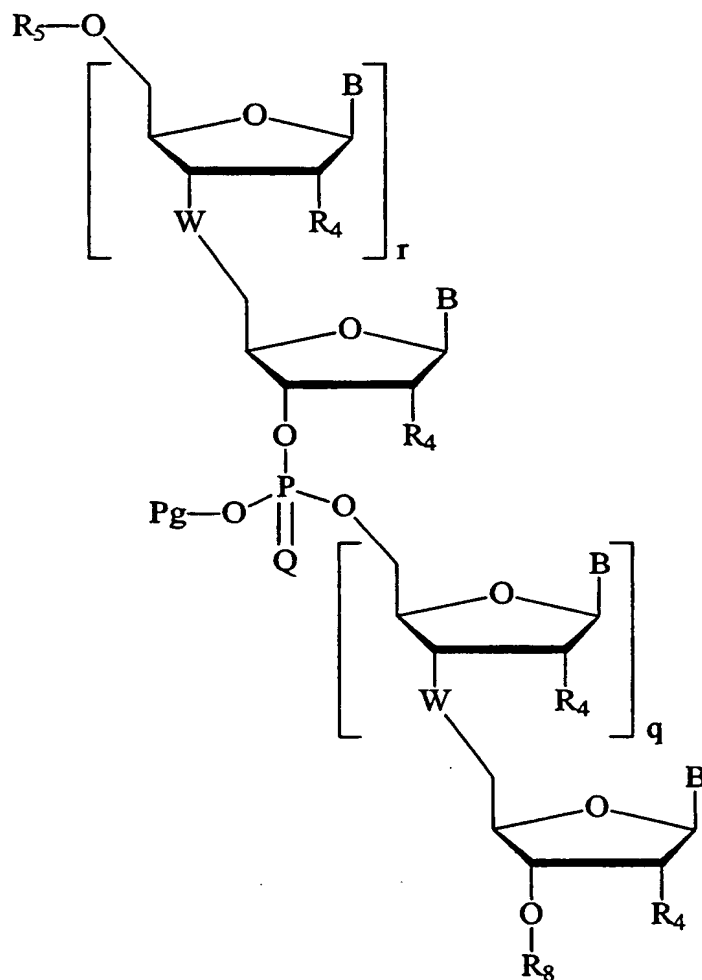
wherein said activating reagent comprises at least one pyridinium salt and one substituted imidazole.

10 Preferably, the activator has the formula G^+U^- , where G^+ is selected from the group consisting of pyridinium, imidazolium, and benzimidazolium; and U^- is selected from the group consisting of hexafluorophosphate, tetrafluoroborate,

triflate, hydrochloride, trifluoroacetate, dichloroacetate, O-mesyl , O-tosyl , Br , and $\text{O-trifluorosulfonyl}$.

Preferably, the compound of Formula XII can then be oxidized or sulfurized to form a compound of Formula

5 XIII:



XIII

where Q is O or S.

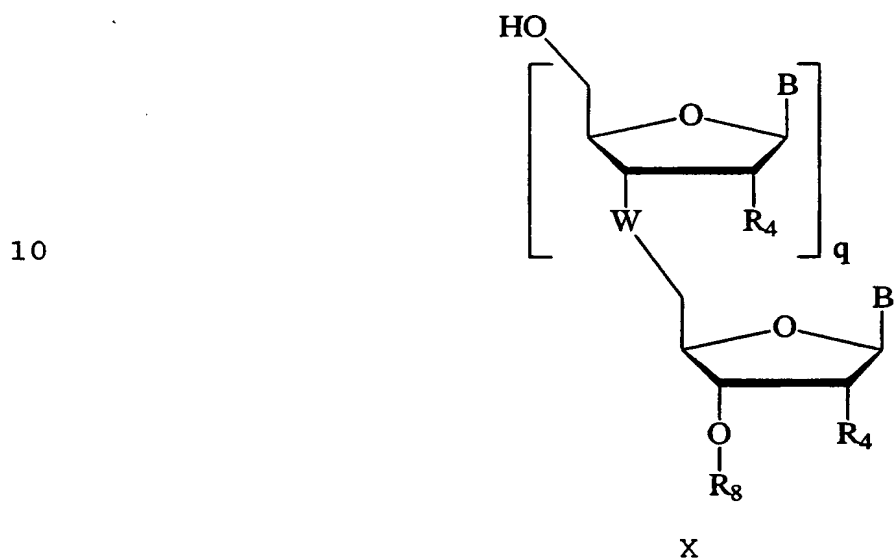
After completion of synthetic regime, the final
10 product is then cleaved from the solid support to produce a
further compound of Formula X.

In a further aspect of the invention, methods are
provided for the preparation of internucleoside linkages
between nucleosides having 2'-substituents, using an
15 imidazolium triflate activating reagent. As used herein,

- 45 -

the term "an imidazolium triflate" denotes imidazolium triflate, as well as substituted imidazolium triflates wherein the substituents are one or more electron withdrawing groups such as, for example, halogen, nitro or cyano. Preferably, the activating reagent is unsubstituted imidazolium triflate. In some preferred embodiments, these methods comprise:

providing a compound of Formula X:



wherein:

B is a nucleobase;

15 R_8 is H, a hydroxyl protecting group, or a linker connected to a solid support;

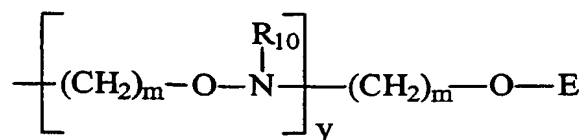
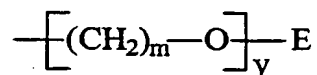
W is an optionally protected internucleoside linkage;

q is 0 to about 50;

R_4 is H, F, O-R, S-R or N-R(R_{10});

20 R is H, a protecting group, or has one of the formulas:

- 46 -



where

each m is independently from 1 to 10;

y is from 0 to 10;

5 E is H, a hydroxyl protecting group, C₁-C₁₀ alkyl, N(R₁₀) (R₁₁) or N=C(R₁₀) (R₁₁); substituted or unsubstituted C₁-C₁₀ alkyl, C₂-C₁₀ alkenyl, C₂-C₁₀ alkynyl, wherein the substitutions are selected from one or several halogen, cyano, carboxy, hydroxy, nitro and mercapto
10 residues; and

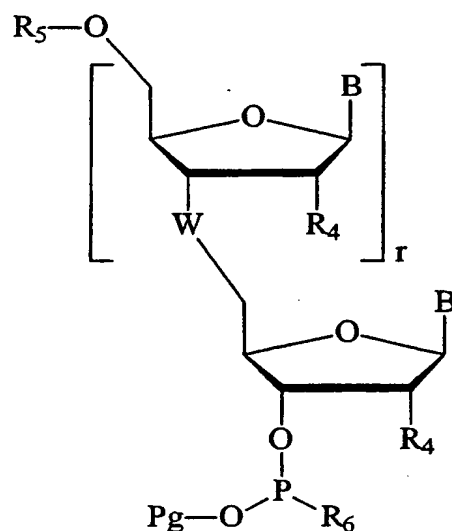
each R₁₀ or R₁₁ is, independently, H, substituted or unsubstituted C₁-C₁₀ alkyl, C₂-C₁₀ alkenyl, C₂-C₁₀ alkynyl, wherein the substitutions are selected from one or several halogen, cyano, carboxy, hydroxy, nitro and mercapto
15 residues; alkylthioalkyl, a nitrogen protecting group, or R₁₀ and R₁₁, together, are a nitrogen protecting group or wherein R₁₀ and R₁₁ are joined in a ring structure that can include at least one heteroatom selected from N and O;

or R is -CH₂-CH₂-O-CH₂-CH₂-N(R₁₀) (R₁₁);

20 provided that R₁₄ is not H or OH;

reacting the compound of Formula X in the presence of an activator with a compound of Formula XI:

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XI

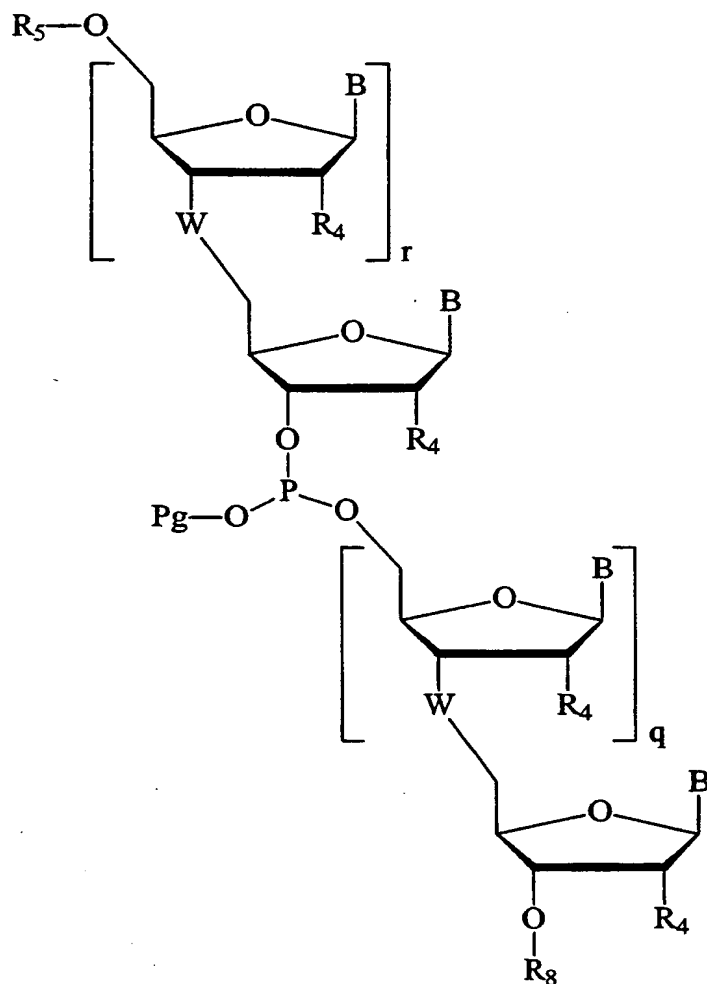
where r is 0 to about 50;

5 R_5 is a hydroxyl protecting group;

R_6 is $-N(R_7)_2$ wherein R_7 is alkyl having from one to about six carbons; or R_7 is a heterocycloalkyl or heterocycloalkenyl ring containing from 4 to 7 atoms, and having up to 3 heteroatoms selected from nitrogen, sulfur,
 10 and oxygen;

to form a compound of Formula XII:

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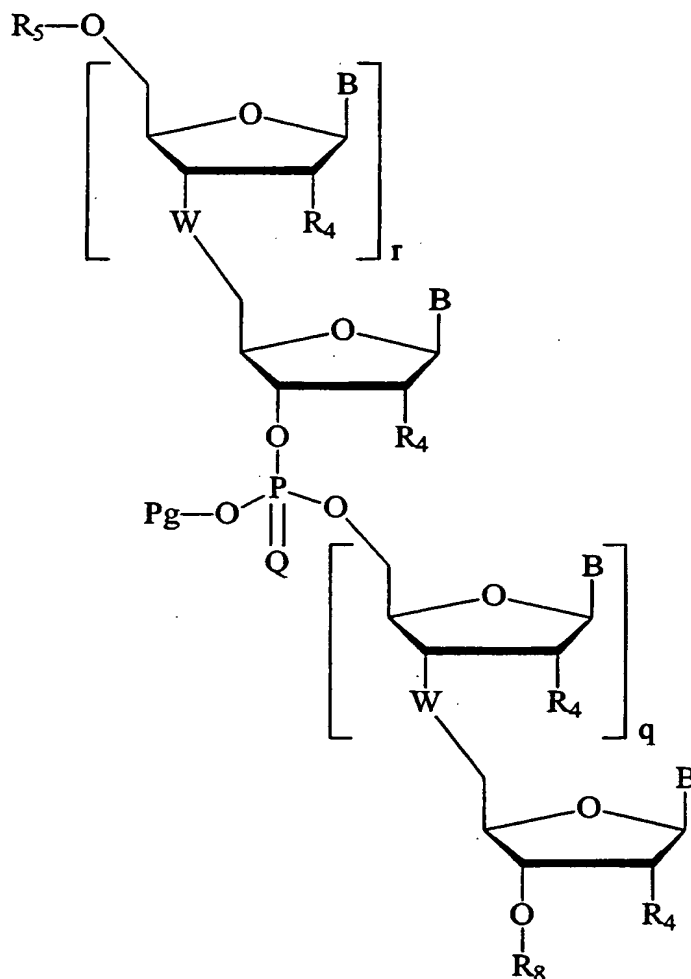


XIII

wherein the activator has the formula G⁺U⁻,
 where G⁺ is selected from the group consisting of pyridinium,
 5 imidazolium, and benzimidazolium; and U⁻ is selected from the
 group consisting of hexafluorophosphate, tetrafluoroborate,
 triflate, hydrochloride, trifluoroacetate, dichloroacetate,
⁻O-mesyl, ⁻O-tosyl, ⁻Br, and ⁻O-trifluorosulfonyl.
 Preferably, the activator is imidazolium triflate.

10 Some further preferred embodiments further
 comprise oxidizing or sulfurizing the compound of Formula
 XII to form a compound of Formula XIII:

- 49 -



XIII

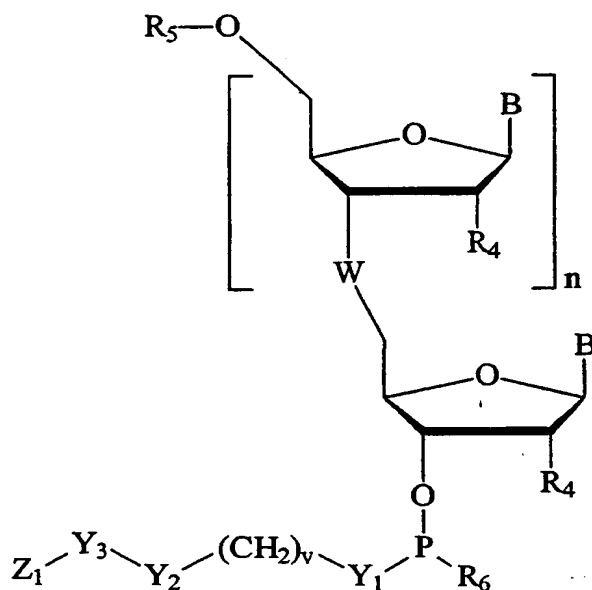
where Q is O or S.

Some further preferred embodiments of the methods
 5 further comprising a capping step, which is preferably
 performed prior to oxidation.

Some further preferred embodiments further
 comprising the step of cleaving the oligomeric compound to
 produce a further compound of formula X.

10 In a further aspect of the invention, synthetic
 methods are provided for the preparation of dimeric and
 higher order oligonucleotides having at least one
 bioreversible protecting group that confers enhanced
 chemical and biophysical properties. In some preferred
 15 embodiments, these methods comprise:
 providing a compound of Formula XX:

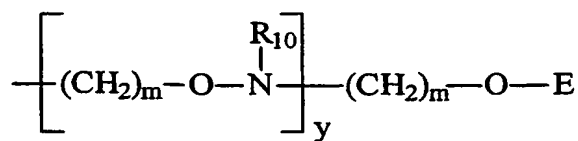
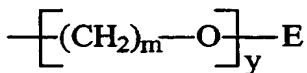
- 50 -



wherein:

R_4 is H, F, O-R, S-R or N-R(R_{10});

5 R is H, a protecting group, or has one of the formulas:



where

each m is independently from 1 to 10;

10 y is from 0 to 10;

E is H, a hydroxyl protecting group, C_1 - C_{10} alkyl, $N(R_{10})(R_{11})$ or $N=C(R_{10})(R_{11})$; substituted or unsubstituted C_1 - C_{10} alkyl, C_2 - C_{10} alkenyl, C_2 - C_{10} alkynyl, wherein the substitutions are selected from one or several
15 halogen, cyano, carboxy, hydroxy, nitro and mercapto residues; and

- 51 -

each R_{10} or R_{11} is, independently, H, substituted or unsubstituted C_1 - C_{10} alkyl, C_2 - C_{10} alkenyl, C_2 - C_{10} alkynyl, wherein the substitutions are selected from one or several halogen, cyano, carboxy, hydroxy, nitro and mercapto residues; alkylthioalkyl, a nitrogen protecting group, or R_{10} and R_{11} , together, are a nitrogen protecting group or wherein R_{10} and R_{11} are joined in a ring structure that can include at least one heteroatom selected from N and O;

or R is $-\text{CH}_2-\text{CH}_2-\text{O}-\text{CH}_2-\text{CH}_2-\text{N}(\text{R}_{10})(\text{R}_{11})$;

10 R_5 is a hydroxyl protecting group;

Z_1 is aryl having 6 to about 14 carbon atoms or alkyl having from one to about six carbon atoms;

Y_1 is O or S;

Y_2 is O or S;

15 Y_3 is $\text{C}(=\text{O})$ or S;

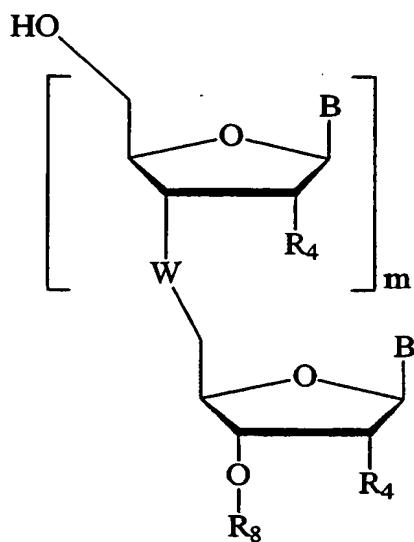
v is 2 to about 4;

B is a nucleobase;

R_6 is $-\text{N}(\text{R}_7)_2$ wherein R_7 is alkyl having from one to about six carbons; or R_7 is a heterocycloalkyl or
20 heterocycloalkenyl ring containing from 4 to 7 atoms, and having up to 3 heteroatoms selected from nitrogen, sulfur, and oxygen;

reacting said compound of Formula XX with a compound of Formula XXI:

- 52 -

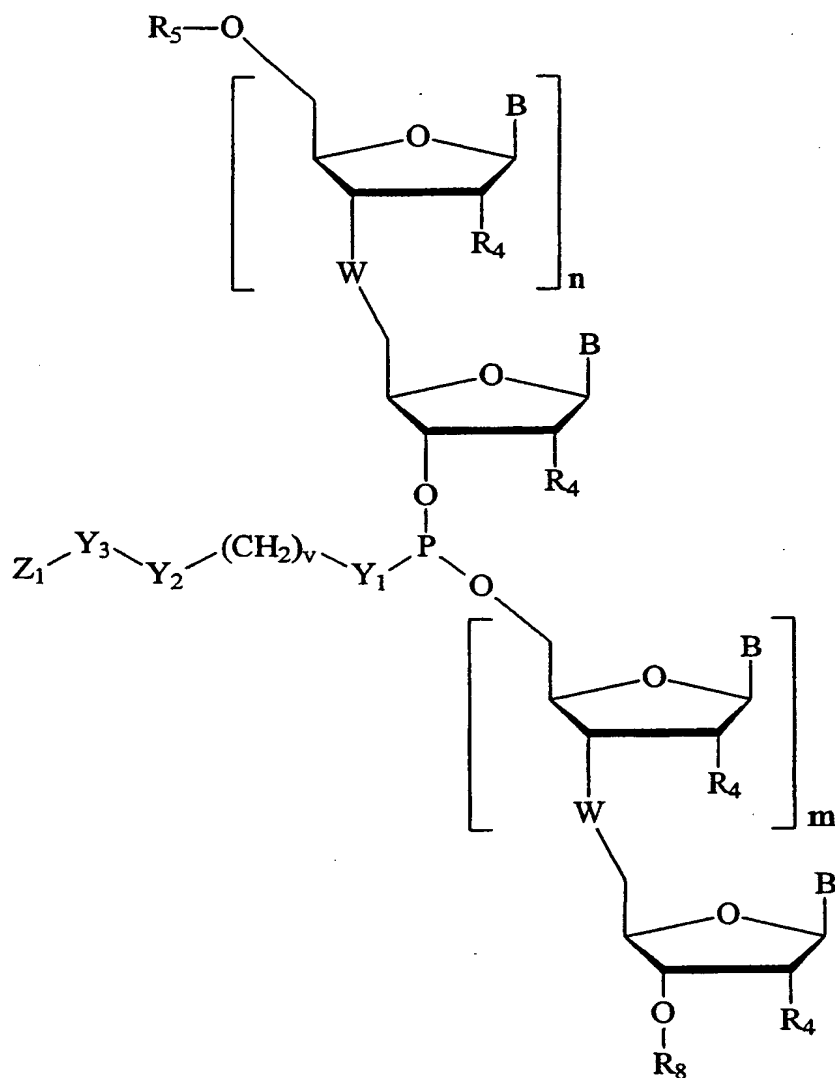


XXI

wherein:

- R_8 is H, a hydroxyl protecting group, or a linker
 5 connected to a solid support;
 in the presence of an activator to form a compound
 of Formula XXII:

- 53 -



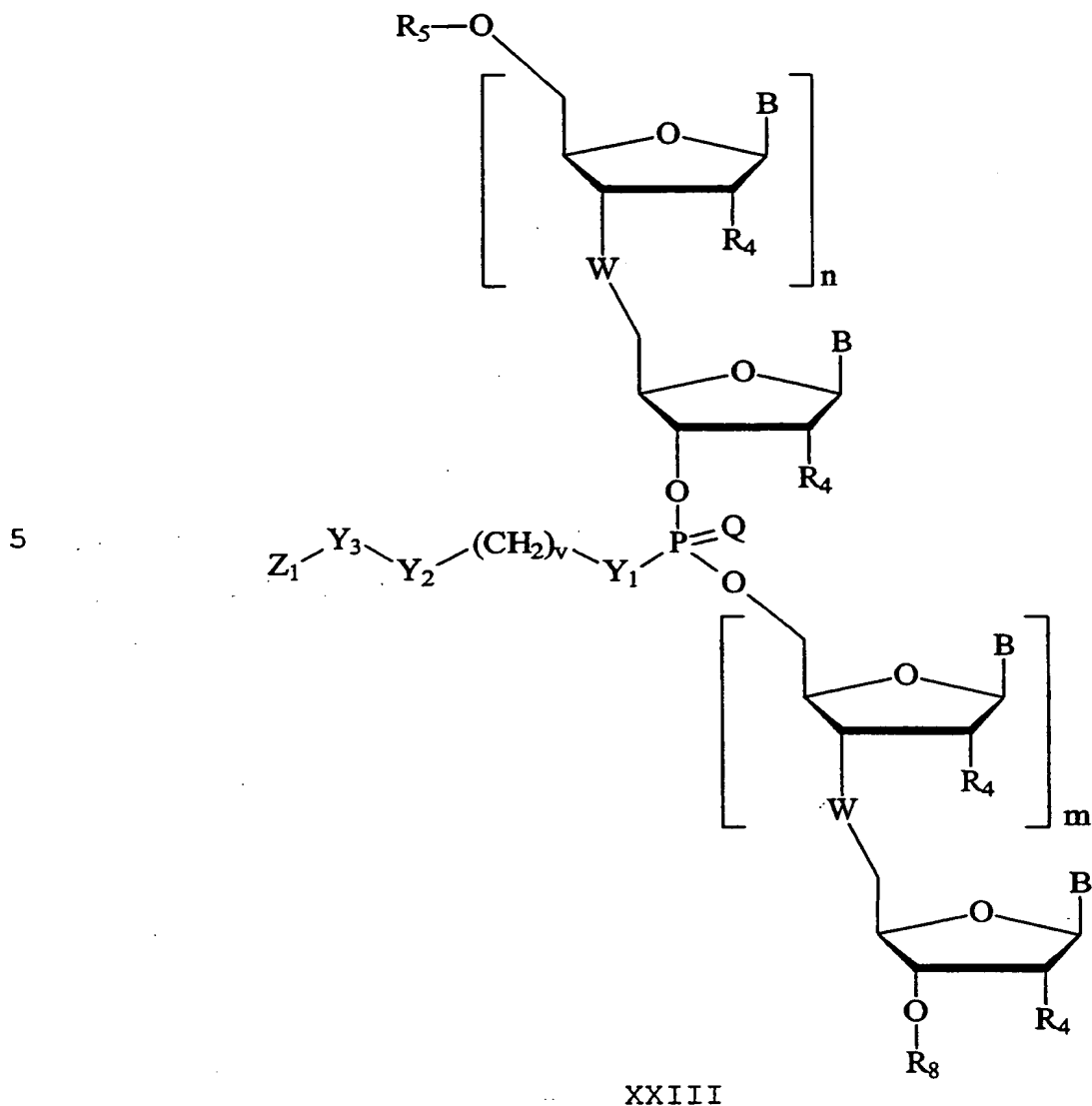
XXII

wherein said activator has the formula G^+U^- , where G^+ is selected from the group consisting of pyridinium, imidazolium, and benzimidazolium; and U^- is selected from the group consisting of hexafluorophosphate, tetrafluoroborate, triflate, hydrochloride, trifluoroacetate, dichloroacetate, ^-O -mesyl, ^-O -tosyl, ^-Br , and ^-O -trifluorosulfonyl. Preferably, the activator is an imidazolium triflate

10 activator.

Some preferred embodiments of the foregoing

methods further comprise oxidizing or sulfurizing the compounds of Formula XXII to form a compound of Formula XXIII:



where Q is O or S.

Some further preferred embodiments of the methods further comprising a capping step, which is preferably performed prior to oxidation.

10

- 55 -

Some further preferred embodiments further comprising the step of cleaving the oligomeric compound to produce a further compound of formula XXI.

Methods for the preparation of compound XX can be
5 found in copending application ser. nos. 09/066,638 and 09/095,822 filed April 24, 1998 and June 11, 1998, respectively, which are assigned to the assignee of the present application. The contents of the foregoing patent applications are hereby incorporated by reference in their
10 entirety.

In further preferred embodiments, each of the foregoing methods, are performed iteratively to produce an oligonucleotide or analog thereof having a preselected nucleotide base sequence. In general, the phosphorus
15 protecting groups, designated "Pg" in the formulas herein, are removed at the end of the synthetic regime, preferably at the time that the completed oligonucleotide or analog is cleaved from the solid support. However, in some preferred embodiments, the methods of the invention are beneficially
20 employed to provide oligonucleotide analogs having at least one bioreversible protecting group that confers enhanced chemical and biophysical properties. See copending applications ser. nos. 09/066,638 and 09/095,822 filed April 24, 1998 and June 11, 1998, respectively. The
25 bioreversible protecting groups further lend nuclease resistance to the oligonucleotides. The bioreversible protecting groups are removed in a cell, in the cell cytosol, or *in vitro* in cytosol extract, by endogenous enzymes. In certain preferred oligonucleotides of the
30 invention the bioreversible protecting groups are designed for cleavage by carboxyesterases to yield unprotected oligonucleotides.

Preferably, the bioreversible protecting group has the Formula $Z_1-Y_3-Y_2-(CH_2)_v-Y_1-$, wherein the constituent
35 variable are as defined above. In some preferred

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embodiments, Y_1 and Y_2 are each O, Y_3 is S, and Z is methyl or t-butyl, with t-butyl being preferred.

One particular advantage of the present invention is that the assembly of oligonucleotides and analogs thereof containing the bioreversible protecting group in accordance with the methods of the invention does not require protection for exocyclic nucleobase amino moieties, thus conferring significant benefit in expense, effort, and yield.

10 In preferred embodiments, the methods of the invention are used for the preparation of oligonucleotides and their analogs. As used herein, the term "oligonucleotide" is intended to include both naturally occurring and non-naturally occurring (i.e., "synthetic")
15 oligonucleotides. Naturally occurring oligonucleotides are those which occur in nature; for example ribose and deoxyribose phosphodiester oligonucleotides having adenine, guanine, cytosine, thymine and uracil nucleobases. As used herein, non-naturally occurring oligonucleotides are
20 oligonucleotides that contain modified sugar, internucleoside linkage and/or nucleobase moieties. Such oligonucleotide analogs are typically structurally distinguishable from, yet functionally interchangeable with, naturally occurring or synthetic wild type oligonucleotides.
25 Thus, non-naturally occurring oligonucleotides include all such structures which function effectively to mimic the structure and/or function of a desired RNA or DNA strand, for example, by hybridizing to a target.

Representative nucleobases include adenine,
30 guanine, cytosine, uridine, and thymine, as well as other non-naturally occurring and natural nucleobases such as xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 5-halo uracil and
35 cytosine, 6-azo uracil, cytosine and thymine, 5-uracil

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(pseudo uracil), 4-thiouracil, 8-halo, oxa, amino, thiol, thioalkyl, hydroxyl and other 8-substituted adenines and guanines, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine. Further naturally and non
5 naturally occurring nucleobases include those disclosed in U.S. Patent No. 3,687,808 (Merigan, et al.), in chapter 15 by Sanghvi, in *Antisense Research and Application*, Ed. S. T. Crooke and B. Lebleu, CRC Press, 1993, in Englisch et al., *Angewandte Chemie*, International Edition, 1991, 30, 613-722
10 (see especially pages 622 and 623, and in the *Concise Encyclopedia of Polymer Science and Engineering*, J.I. Kroschwitz Ed., John Wiley & Sons, 1990, pages 858-859, Cook, *Anti-Cancer Drug Design* 1991, 6, 585-607, each of which are hereby incorporated by reference in their
15 entirety). The term "nucleosidic base" is further intended to include heterocyclic compounds that can serve as like nucleosidic bases including certain "universal bases" that are not nucleosidic bases in the most classical sense but serve as nucleosidic bases. Especially mentioned as a
20 universal base is 3-nitropyrrole.

Representative 2' sugar modifications (moiety R₁ in the formulas described herein) amenable to the present invention include fluoro, O-alkyl, O-alkylamino, O-alkylalkoxy, protected O-alkylamino, O-alkylaminoalkyl, O-
25 alkyl imidazole, and polyethers of the formula (O-alkyl)_m, where m is 1 to about 10. Preferred among these polyethers are linear and cyclic polyethylene glycols (PEGs), and (PEG)-containing groups, such as crown ethers and those which are disclosed by Ouchi, et al., *Drug Design and*
30 *Discovery* 1992, 9, 93, Ravasio, et al., *J. Org. Chem.* 1991, 56, 4329, and Delgado et. al., *Critical Reviews in Therapeutic Drug Carrier Systems* 1992, 9, 249, each of which are hereby incorporated by reference in their entirety. Further sugar modifications are disclosed in Cook, P.D.,

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supra. Fluoro, O-alkyl, O-alkylamino, O-alkyl imidazole, O-alkylaminoalkyl, and alkyl amino substitution is described in United States Patent Application serial number 08/398,901, filed March 6, 1995, entitled Oligomeric

5 Compounds having Pyrimidine Nucleotide(s) with 2' and 5' Substitutions, hereby incorporated by reference in its entirety.

Sugars having O-substitutions on the ribosyl ring are also amenable to the present invention. Representative

10 substitutions for ring O include S, CH₂, CHF, and CF₂, see, e.g., Secrist, et al., *Abstract 21, Program & Abstracts, Tenth International Roundtable, Nucleosides, Nucleotides and their Biological Applications*, Park City, Utah, Sept. 16-20, 1992, hereby incorporated by reference in its entirety.

15 As used herein, the term "alkyl" includes but is not limited to straight chain, branch chain, and alicyclic hydrocarbon groups. Alkyl groups of the present invention may be substituted. Representative alkyl substituents are disclosed in United States Patent No. 5,212,295, at column

20 12, lines 41-50, hereby incorporated by reference in its entirety.

"Aryl" groups are aromatic cyclic compounds including but not limited to phenyl, naphthyl, anthracyl, phenanthryl, pyrenyl, and xylyl.

25 In general, the term "hetero" denotes an atom other than carbon, preferably but not exclusively N, O, or S. Accordingly, the term "heterocycloalkyl" denotes an alkyl ring system having one or more heteroatoms (i.e., non-carbon atoms). Preferred heterocycloalkyl groups include,

30 for example, morpholino groups. As used herein, the term "heterocycloalkenyl" denotes a ring system having one or more double bonds, and one or more heteroatoms. Preferred heterocycloalkenyl groups include, for example, pyrrolidino groups.

35 In some preferred embodiments of the invention R₈

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can be a linker connected to a solid support. Solid supports are substrates which are capable of serving as the solid phase in solid phase synthetic methodologies, such as those described in Caruthers U.S. Patents Nos. 4,415,732; 4,458,066; 4,500,707; 4,668,777; 4,973,679; and 5,132,418; and Koster U.S. Patents Nos. 4,725,677 and Re. 34,069.

Linkers are known in the art as short molecules which serve to connect a solid support to functional groups (e.g., hydroxyl groups) of initial synthon molecules in solid phase synthetic techniques. Suitable linkers are disclosed in, for example, *Oligonucleotides And Analogues A Practical Approach*, Ekstein, F. Ed., IRL Press, N.Y, 1991, Chapter 1, pages 1-23, hereby incorporated by reference in its entirety.

Preferred linkers for use in linking the growing oligonucleotide chain to the solid support in some preferred embodiments of the methods of the invention will be cleaved by reagents that do not result in removal of the $-Y_1-(CH_2)_q-Y_2-Y_3-Z$ protecting group. One such linker is the oxalyl linker (Alul, R.H., et al., *Nucl. Acids Res.* 1991, 19, 1527) between a LCAA-CPG solid support and the oligomer. Other photolabile supports have been reported (Holmes et al., *J. Org. Chem.* 1997, 62, 2370-2380; Greenberg et al., *Tetrahedron Lett.* 1993, 34, 251-254). The o-nitrobenzyl functionalized solid support has been previously reported (Dell'Aquila et al., *Tetrahedron Lett.* 1997, 38, 5289-5292). Another preferred method of cleavage without removal of internucleoside protecting groups is by irradiation with ultraviolet light in aqueous acetonitrile.

Solid supports according to the invention include those generally known in the art to be suitable for use in solid phase methodologies, including, for example, controlled pore glass (CPG), oxalyl-controlled pore glass (see, e.g., Alul, et al., *Nucleic Acids Research* 1991, 19,

1527, hereby incorporated by reference in its entirety),
TentaGel Support, an aminopolyethyleneglycol derivatized
support (see, e.g., Wright, et al., *Tetrahedron Letters*
1993, 34, 3373, hereby incorporated by reference in its
5 entirety) and Poros, a copolymer of
polystyrene/divinylbenzene.

In some preferred embodiments of the invention R_5
or R_6 can be a hydroxyl protecting group. A wide variety of
hydroxyl protecting groups can be employed in the methods of
10 the invention. Preferably, the protecting group is stable
under basic conditions but can be removed under acidic
conditions. In general, protecting groups render chemical
functionalities inert to specific reaction conditions, and
can be appended to and removed from such functionalities in
15 a molecule without substantially damaging the remainder of
the molecule. Representative hydroxyl protecting groups are
disclosed by Beaucage, et al., *Tetrahedron* 1992, 48, 2223-
2311, and also in Greene and Wuts, *Protective Groups in*
Organic Synthesis, Chapter 2, 2d ed, John Wiley & Sons, New
20 York, 1991, each of which are hereby incorporated by
reference in their entirety. Preferred protecting groups
used for R_5 and R_6 include dimethoxytrityl (DMT),
monomethoxytrityl, 9-phenylxanthen-9-yl (Pixyl) and 9-(p-
methoxyphenyl)xanthen-9-yl (Mox). The R_5 or R_6 group can be
25 removed from oligomeric compounds of the invention by
techniques well known in the art to form the free hydroxyl.
For example, dimethoxytrityl protecting groups can be
removed by protic acids such as formic acid, dichloroacetic
acid, trichloroacetic acid, p-toluene sulphonic acid or with
30 Lewis acids such as for example zinc bromide. See for
example, Greene and Wuts, *supra*.

In some preferred embodiments of the invention
amino groups are appended to alkyl or other groups, such as,
for example, 2'-alkoxy groups (e.g., where R_1 is alkoxy).
35 Such amino groups are also commonly present in naturally

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occurring and non-naturally occurring nucleobases. It is generally preferred that these amino groups be in protected form during the synthesis of oligomeric compounds of the invention. Representative amino protecting groups suitable for these purposes are discussed in Greene and Wuts, *Protective Groups in Organic Synthesis*, Chapter 7, 2d ed, John Wiley & Sons, New York, 1991. Generally, as used herein, the term "protected" when used in connection with a molecular moiety such as "nucleobase" indicates that the molecular moiety contains one or more functionalities protected by protecting groups.

Sulfurizing agents used during oxidation to form phosphorothioate and phosphorodithioate linkages include Beaucage reagent (see e.g. Iyer, et.al., *J. Chem. Soc.* 1990, 112, 1253-1254, and Iyer, et.al., *J. Org. Chem.* 1990, 55, 4693-4699); tetraethylthiuram disulfide (see e.g., Vu, et al., *Tetrahedron Lett.* 1991, 32, 3005-3008); dibenzoyl tetrasulfide (see e.g., Rao, et.al., *Tetrahedron Lett.* 1992, 33, 4839-4842); di(phenylacetyl)disulfide (see e.g., Kamer, *Tetrahedron Lett.* 1989, 30, 6757-6760); Bis(O,O-diisopropoxy phosphinothioyl)disulfide (see Stec et al., *Tetrahedron Lett.* 1993, 34, 5317-5320); 3-ethoxy-1,2,4-dithiazoline-5-one (see *Nucleic Acids Research*, 1996 24, 1602-1607, and *Nucleic Acids Research*, 1996 24, 3643-3644); Bis(p-chlorobenzenesulfonyl)disulfide (see *Nucleic Acids Research*, 1995 23, 4029-4033); sulfur, sulfur in combination with ligands like triaryl, trialkyl, triaralkyl, or trialkaryl phosphines. The foregoing references are hereby incorporated by reference in their entirety.

Useful oxidizing agents used to form the phosphodiester or phosphorothioate linkages include iodine/tetrahydrofuran/ water/pyridine or hydrogen peroxide/water or tert-butyl hydroperoxide or any peracid like m-chloroperbenzoic acid. In the case of sulfurization

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the reaction is performed under anhydrous conditions with the exclusion of air, in particular oxygen whereas in the case of oxidation the reaction can be performed under aqueous conditions.

5 Oligonucleotides or oligonucleotide analogs according to the present invention hybridizable to a specific target preferably comprise from about 5 to about 50 monomer subunits. It is more preferred that such compounds
10 comprise from about 10 to about 30 monomer subunits, with 15 to 25 monomer subunits being particularly preferred. When used as "building blocks" in assembling larger oligomeric compounds (*i.e.*, as synthons of Formula II), smaller oligomeric compounds are preferred. Libraries of dimeric, trimeric, or higher order compounds of general Formula II
15 can be prepared for use as synthons in the methods of the invention. The use of small sequences synthesized via solution phase chemistries in automated synthesis of larger oligonucleotides enhances the coupling efficiency and the purity of the final oligonucleotides. See for example:
20 Miura, *et al.*, *Chem. Pharm. Bull.* **1987**, *35*, 833-836; Kumar, *et al.*, *J. Org. Chem.* **1984**, *49*, 4905-4912; Bannwarth, *Helvetica Chimica Acta* **1985**, *68*, 1907-1913; Wolter, *et al.*, *Nucleosides and Nucleotides* **1986**, *5*, 65-77, each of which are hereby incorporated by reference in their entirety.

25 The oligonucleotides produced by preferred embodiments of the methods of the invention having bioreversible protecting groups are also referred to in this specification as pro-oligonucleotides. Such pro-oligonucleotides are capable of improved cellular lipid
30 bilayers penetrating potential as well as resistance to exo- and endonuclease degradation *in vivo*. In cells, the bioreversible protecting groups are removed in the cell cytosol by endogenous carboxyesterases to yield biologically active oligonucleotide compounds that are capable of
35 hybridizing to and/or having an affinity for specific

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nucleic acid.

Additional advantages and novel features of this invention will become apparent to those skilled in the art upon examination of the examples thereof provided below, which should not be construed as limiting the appended claims.

Preparation of Phosphoramidites

Example 1

General phosphitylation procedure using 2'-deoxy-5'-O-DMT nucleosides with pyridinium trifluoroacetate

To a sample of 2'-deoxy-5'-O-DMT-nucleoside (2'-O-deoxy-5'-O-DMT-6-N-benzoyladenine, 2'-O-deoxy-5'-O-DMT-4-N-benzoylcytidine, 2'-O-deoxy-5'-O-DMT-2-N-isobutyrylguanosine and 2'-O-deoxy-5'-O-DMT-thymidine) (1a-1d, Figure 1, 10 mmol, 5.45 - 6.40 g) in dry dichloromethane (25 mL) was added bisamidite reagent (2-cyanoethyl-N,N,N',N'-tetraisopropylphosphorodiamidite, 5, figure 1, 3.81 mL, 3.62 g, 12 mmol) at ambient temperature under argon. Pyridinium trifluoroacetate (2.32 g, 12 mmol) was added and the reaction mixture was stirred at ambient temperature for 2-3 hours. The reaction was diluted with dichloromethane (35 mL), and washed with of saturated NaHCO₃/H₂O (30 mL). The organic layer was separated, dried (Na₂SO₄), evaporated, and purified on a short silica gel column. The amidite product was eluted with 60-80% EtOAc/hexanes (1% triethylamine) with the exact concentration dependent to the respective amidite being purified. The appropriate fractions were collected and evaporated to give the respective amidite product (6a-d, Figure 1) as a white foam in ~ 80% yield.

Example 2

General phosphitylation procedure using 2'-O-methyl-5'-O-DMT nucleosides with pyridinium trifluoroacetate

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To a sample of 2'-O-methyl-5'-O-DMT-nucleoside (2'-O-methyl-5'-O-DMT-6-N-benzoyladenosine, 2'-O-methyl-5'-O-DMT-4-N-benzoylcytidine, 2'-O-methyl-5'-O-DMT-2-N-isobutyrylguanosine, 2'-O-methyl-5'-O-DMT-thymidine and 2'-O-methyl-5'-O-DMT-uridine) (2a-2e, Figure 1, 1 mmol, 560 - 670 mg) in dry dichloromethane (3 mL) was added bisamidite reagent (0.38 mL, 362 mg, 1.2 mmol) at ambient temperature under argon. Pyridinium trifluoroacetate (232 mg, 1.2 mmol) was added to the reaction flask and the reaction mixture was stirred at ambient temperature for 2-3 hours. The reaction mixture was transferred directly to the top of a short silica gel column. The amidite product was eluted with 60-80% EtOAc/hexanes (1% triethylamine) with the exact concentration dependent to the respective amidite being purified. The appropriate fractions were collected and evaporated to give the respective amidite product (7a-e, Figure 1) as a white foam in 75-94% yield.

Example 3

General phosphitylation procedure using 2'-O-TBDMS-5'-O-DMT nucleosides with pyridinium trifluoroacetate

To a sample of 2'-O-TBDMS-5'-O-DMT-nucleoside (2'-O-TBDMS-5'-O-DMT-6-N-benzoyladenosine, 2'-O-TBDMS-5'-O-DMT-4-N-benzoylcytidine, 2'-O-TBDMS-5'-O-DMT-2-N-isobutyrylguanosine, 2'-O-TBDMS-5'-O-DMT-thymidine and 2'-O-TBDMS-5'-O-DMT-uridine) (3a-3e, Figure 1, 1 mmol, 661 - 770 mg) in dry dichloromethane (3 mL) was added bisamidite reagent (0.38 mL, 362 mg, 1.2 mmol) at ambient temperature under argon. Pyridinium trifluoroacetate (232 mg, 1.2 mmol) was added to the reaction flask and the reaction mixture was stirred at ambient temperature for 2-3 hours. The reaction solution was transferred directly to the top of a short silica gel column. The amidite product was eluted with 45-60% EtOAc/hexanes (1% triethylamine) with the exact concentration dependent to the respective amidite being

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purified. The appropriate fractions were collected and evaporated to give the respective amidite product (8a-e, Figure 1) as a white foam in 82-95% yield.

5 Example 4

General phosphitylation procedure using 2'-O-methoxyethyl-5'-O-DMT nucleosides with pyridinium trifluoroacetate

To a sample of 2'-O-(2-methoxyethyl)-5'-O-DMT-nucleoside (2'-O-(2-methoxyethyl)-5'-O-DMT-6-N-benzoyladenine, 2'-O-(2-methoxyethyl)-5'-O-DMT-4-N-benzoylcytidine, 2'-O-(2-methoxyethyl)-5'-O-DMT-2-N-isobutyrylguanosine, 2'-O-(2-methoxyethyl)-5'-O-DMT-thymidine, 2'-O-(2-methoxyethyl)-5'-O-DMT-uridine and 5-methyl-2'-O-(2-methoxyethyl)-5'-O-DMT-4-N-benzoylcytidine) (4a-f, Figure 1, 1 mmol, 619 - 714 mg) in dry dichloromethane (3 mL) was added bisamidite reagent (0.38 mL, 362 mg, 1.2 mmol) at ambient temperature under argon. Pyridinium trifluoroacetate (232 mg, 1.2 mmol) was added to the reaction flask and the reaction mixture was stirred at ambient temperature for 2-3 h. The reaction solution was transferred directly to the top of a short silica gel column. The amidite product was eluted with 60-80% EtOAc/hexanes (1% triethylamine) with the exact concentration dependent to the respective amidite being purified. The appropriate fractions were collected and evaporated to give the respective amidite product (9a-f, Figure 1) as a white foam in 92-95% yield.

Example 5

30 General procedure for phosphitylation of nucleoside 2'-deoxy-5'-O-DMT-4-N-benzoylcytidine with Poly(4-vinyl pyridine hydrochloride) as an activator

Poly(4-vinyl pyridine hydrochloride) (Aldrich, 583 mg, ~ 6.5 mmol Cl/g) resin was washed with dry acetonitrile

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(10 mL x 2). Dry dichloromethane (15 mL) and bisamidite reagent (1.20 mL, 1.14 g, 3.79 mmol) were added to the resin at ambient temperature under argon. Then a sample of 2'-deoxy-5'-O-DMT-4-N-benzoylcytidine (2.0 g, 3.16 mmol) was added and the reaction mixture was shaken by a mechanical shaker for 2 hours. The reaction was filtered and the filtrate was evaporated, and the residue was purified on a short silica gel column. The amidite product was eluted with 60% EtOAc/hexanes (1% triethylamine). The appropriate fractions were collected and evaporated to give amidite product (6b, figure 1) as a white foam (369 mg, 14%).

³¹P NMR (CDCl₃) δ 149.34, 149.94.

Example 6

General procedure for phosphitylation of the 5'-O-position of 6-N-benzoyl-2'-deoxy-3'-O-levulinyladenosine using pyridinium trifluoroacetate

A sample of 6-N-benzoyl-2'-deoxy-3'-O-levulinyladenosine (10, Figure 1, 1 mmol, 453 mg) in dry dichloromethane (3 mL) was added bisamidite reagent (0.38 mL, 362 mg, 1.2 mmol) at ambient temperature under argon. Pyridinium trifluoroacetate (232 mg, 1.2 mmol) was added and the reaction mixture was stirred at ambient temperature for 2 hours. The reaction solution was transferred directly to the top of a short silica gel column. The amidite product was eluted with EtOAc (1% triethylamine). The appropriate fractions were collected and evaporated to give the amidite product (11, Figure 1) as a white foam (601 mg, 92%).

³¹P NMR (CDCl₃) δ 149.58.

Example 7

Comparative study of activator efficiency using a) pyridinium acetate, b) pyridinium monochloroacetate, c) pyridinium dichloroacetate and d) pyridinium

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trichloroacetate

Four separate reactions were run to determine the efficiency of selected pyridinium salts to act as an activator in phosphitylating 2'-deoxy-5'-O-DMT-4-N-benzoylcytidine. The activator species were produced *in situ* by addition of 1.2 eq. of the corresponding acetic acid, mono-, di- or trichloroacetic acid (0.56 mmol) to dry dichloromethane (1.5 mL) followed by addition of 1.3 eq. of pyridine (0.049 mL, 0.61 mmol). Bisamidite reagent (0.177 mL, 0.56 mmol) and 2'-deoxy-5'-O-DMT-4-N-benzoylcytidine (300 mg, 0.47 mmol) were added and the reaction mixtures were stirred under argon at ambient temperature. The progress of the reactions was monitored by TLC. There was no measurable reaction seen with the use of acetic acid and a slow reaction by use of either mono- or trichloroacetic acid (reaction not finished after 6.5 hours). At 6.5 hours the reaction was almost complete when dichloroacetic acid was used.

Example 8

Phosphitylation using pyridinium dichloroacetate, synthesis of 2'-deoxy-5'-O-DMT-4-N-benzoylcytidine diisopropylamino-cyanoethoxyphosphoramidite

Pyridinium dichloroacetate was prepared *in situ* by addition of 1.3 equivalents of pyridine (0.49 mL, 6.07 mmol) to dry dichloromethane (4 mL) followed by addition of 1.2 eq. of dichloroacetic acid (0.46 mL, 5.60 mmol). To this mixture was added bisamidite reagent (1.78 mL, 5.60 mmol) followed by the dropwise addition of 2'-deoxy-5'-O-DMT-4-N-benzoylcytidine (2.97 g, 4.67 mmol) dissolved in dry dichloromethane (6 mL). The reaction mixture was stirred under argon at ambient temperature for 2 hours and transferred directly to the top of a short silica gel column. The amidite product was eluted with 70%

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EtOAc/hexanes (1% triethylamine). The appropriate fractions were collected and evaporated to give 3.47 g (89%) of the title compound as a white foam.

^{31}P NMR (CDCl_3) δ 149.29, 149.88.

5 Example 9

Stability of 5'-O-DMT protecting group to reaction conditions, synthesis of 2'-deoxy-5'-O-DMT-4-N-benzoylcytidine diisopropylaminocyanoethoxyphosphoramidite

4-N-Benzoyl-2'-deoxy-5'-O-DMT-cytidine (1.77 g, 2.79 mmol) was dissolved in dry dichloromethane (4 mL) under argon at ambient temperature followed by addition of bisamidite reagent (1.06 mL, 3.35 mmol) and pyridinium trifluoroacetate (0.65 g, 3.35 mmol). The mixture was stirred under reflux for 5 hours with no measurable loss of DMT protecting group. Product formation was identified by tlc compared to a known solution of product.

Example 10

Preparation of amidites without base protection, synthesis of 2'-deoxy-5'-O-DMT-adenosine diisopropylaminocyanoethoxyphosphoramidite

Pyridinium trifluoroacetate (353 mg, 1.83 mmol) was added to a mixture of 2'-deoxy-5'-O-DMT-adenosine (1g, 841 mg, 1.52 mmol) and bisamidite reagent (0.53 mL, 505 mg, 1.67 mmol) in dichloromethane (5 mL). Stirring was continued for one hour at ambient temperature under argon atmosphere. The reaction solution was loaded without further workup on a silica gel column and eluted using a gradient of from 60 to 100% EtOAc/hexanes (1% triethylamine). The appropriate fractions were collected and evaporated to give 6.0g of the title compound as a white foam (689 mg, 60%).

^{31}P NMR (CDCl_3) δ 149.26, 149.92.

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Example 11

**Stability of glycosidic linkage to reaction conditions,
synthesis of 2'-deoxy-5'-O-DMT-6-N-benzoyladenosine
diisopropylaminocynoethoxyphosphoramidite**

- 5 2'-deoxy-5'-O-DMT-6-N-benzoyladenosine Procedure
(500 mg, 0.76 mmol) was dissolved in dry dichloromethane (1
mL) under argon at ambient temperature followed by addition
of bisamidite reagent (0.266 mL, 252mg, 0.837 mmol) and
pyridinium trifluoroacetate (176 mg, 0.913 mmol). The
10 mixture was stirred at ambient temperature for 2 hours, and
then stirred under reflux for 1.5 hours with no measurable
loss of the DMT protecting group or the adenine base.
Product formation was identified by tlc compared to a known
solution of product. This example shows the stability of
15 the most labile glycosidic linkage of a nucleoside under the
reaction conditions using this activator.

Example 12

**Mechanistic study of phosphitylation using pyridinium
trifluoroacetate**

- 20 The mechanism of phosphitylation was investigated
using the activator pyridinium trifluoroacetate and the
nucleoside 5'-O-DMT-thymidine using a Varian 400 MHz NMR.
The first set of experiments were performed by studying the
chemical shift of phosphorus nuclei under various conditions
25 (Table 1). In a second set of experiments the chemical
shift of nitrogen nuclei of various species were studied
(Table 2).

- The presence or absence of a specific phosphorus
species was determined by recording ^{31}P NMR of 5'-O-DMT-
30 thymidine, bisamidite reagent and pyridinium
trifluoroacetate in CD_3CN . The order of addition was altered
in each individual experiment to determine which species is
formed in the reaction mixture.

In experiment no. 1 (Table 1) the chemical shift

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of the ^{31}P signal in bisamidite reagent (5) is measured to be at 125.8 ppm in CD_3CN . The activator pyridinium trifluoroacetate (B) is then added to the solution of the solution of 5 and the ^{31}P NMR was recorded. A new signal appeared at 158.8 ppm upon addition of B to 5, in addition to the original signal of 125.8 ppm. The peak at 158 is believed to be a protonated species of 5 which appears to be stable and formed quickly. Next, addition of 5'-O-DMT-thymidine (1d) to the mixture shifts the signals to 151.2 and 151.0 ppm, due to the formation of diastereoisomers.

In experiment no. 2 (Table 1) 5'-O-DMT-thymidine (1d) bisamidite reagent (B) were taken together in CD_3CN and the ^{31}P NMR was recorded. It is note worthy that B alone can not react because the reagent is not activated or protonated. Thus, the chemical shift remains unchanged at 125.8 ppm. Addition of activator B to the mixture immediately forms the desired amidite 6d with ^{31}P shifts of 151.2 and 151.0 ppm.

In another experiment, bisamidite reagent (5) was treated with an acid such as trifluoroacetic acid instead of activator B and the ^{31}P NMR was recorded. First, the color of the reaction mixture changed from clear to dark and second there was no signal at 158 ppm for the protonated species.

Table 1

<u>compound</u>	<u>^{31}P NMR, chemical shift ppm (multiplicity)</u>
<u>Exp. No 1</u>	
5	125.8 (s)
5+B	158.8 (s)
	125.8 (s)
5+B+1d	151.2, 151.0 (s+s)

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Exp. No 2

1d+5	125.8(s)
1d+5+B	151.2, 151.0 (s+s)
5	125.8(s)

Wherein 1d = 2'-O-deoxy-5'-O-DMT-thymidine
5 = 2-cyanoethyl-N,N,N',N'-tetraiso
propylphosphorodiamidite

10 B = pyridinium trifluoroacetate

The experimental data show that the protonation of the phosphitylating reagent is the first step during the reaction sequence and that the protonated form is stable.

15 Support for this conclusion comes from the lack of signals seen for a second activated species that could form from nucleophilic attack by the counterion on the active species or alternatively reaction with free pyridine released during the protonation step. It is further seen that the use of
20 trifluoroacetic acid alone results in degradation of the phosphitylating reagent. If an acid was all that was needed for activation of the phosphitylating reagent then the active phosphorous species should be formed anyway, with a possible following attack of the trifluoroacetate. These
25 results demonstrate that the counterion does not participate in the mechanism. In other words the pyridinium ion acts as a proton donor and does not interact with the active phosphorus species further.

In experiment no. 3, (Table 2) ¹⁵N-labeled pyridine
30 was used to further establish the role of the free pyridine formed during the activation of the phosphitylating reagent. It had previously been seen that a phosphorus species having pyridine acting to give nucleophilic assistance was not seen.

Table 2

<u>compound</u>	<u>¹⁵N NMR, chemical shift ppm</u>
<u>Exp. No 3</u>	
12	-69.0
12+13	-148.0
5+12+13	-68.5
1d+5+12+13	-71.74

10 Wherein 1d = 2'-O-deoxy-5'-O-DMT-thymidine
 5 = 2-cyanoethyl-N,N,N',N'-tetraiso-
 propylphosphorodiamidite
 12 = pyridine
 13 = trifluoroacetic acid

15

The ¹⁵N-labeled pyridine alone gives a signal at -
 69.0 ppm. The *in situ* formation of the activator is
 performed by addition of the trifluoroacetic acid. A signal
 is seen for the activator (pyridinium trifluoroacetate) at -
 20 148.0 ppm. Next, addition of the bisamidite reagent (5)
 causes the signal of the pyridine to revert back to that of
 free pyridine as expected. Again, addition of 2'-O-deoxy-
 5'-O-DMT-thymidine (1d) which underwent phosphitylation did
 not change the free pyridine signal. In conclusion,
 25 pyridine reacts with the trifluoroacetic acid to form
 activator (B) that reacts with 5 to produce activated
 phosphitylating reagent. At this point the pyridine reverts
 back to free pyridine where it remains unchanged for the
 remainder of the phosphitylation reaction. The slight
 30 change in chemical shift (Table 2) after the addition of the
 other reagents is due to the extreme sensitivity of the
 nitrogen signal to the concentration.

Example 13

Determination of efficiency of selected activators

In order to determine the efficiency of activators under a variety of conditions 55 experiments were performed using a wide range of different activators (see Figures 2 and 3). A variety of nucleosides were employed including 2'-deoxy and 2'-O-modified nucleosides (see Figure 1). Different solvents were also employed including a dichloromethane, acetonitrile, ethyl acetate and toluene to evaluate the rate of reaction.

Table 3

<u>Cmpd.Act.</u>		<u>Ratio</u>	<u>Sol</u>	<u>Time</u>	<u>Yld.</u>	<u>³¹P NMR</u>
<u>I</u>	<u>II</u>	<u>(I:P[III]:II)</u>			<u>hrs.</u>	<u>(%)</u>
15	1a	A	1 : 1.4 : 1.4	DCM	3	72 149.32, 149.43
	1a	H	1 : 1.2 : 1.2	DCM	3	54 149.32, 149.43
	1a	F	1 : 1.2 : 1.2	ACN	2	51 149.32, 149.43
	1a	F	1 : 0.7 : 1.2	ACN	4.5	52 149.32, 149.43
	1a	C	1 : 1.2 : 1	ACN	2	42 149.32, 149.43
20	1a	B	1 : 1.2 : 1.2	ACN	2	90 149.32, 149.43
	1a	B	1 : 1.2 : 1.2	DCM	2	68 149.32, 149.43
	1a	I	1 : 1.2 : 1.2	ACN	48	- N/A
	1a	I	1 : 1.2 : 1.2	DCM	48	- N/A
	1a	J	1 : 1.2 : 1.2	DCM	48	- N/A
25	1a	K	1 : 1.2 : 1.2	DCM	17.5	- N/A
	1a	L	1 : 1.2 : 1.2	ACN	17.5	- N/A
	1a	M	1 : 1.2 : 1.2	DCM	0.25	71 149.32, 149.43
	1a	N	1 : 1.2 : 1.2	DCM	3	35 149.32, 149.43
	1a	N	1 : 1.2 : 1.2	DCM	20	32 149.32, 149.43
30	1b	A	1: 1.2 : 1.2	DCM	4	87 149.29, 149.88
	1b	B	1: 1.2 : 1.2	ACN	5	74 149.29, 149.88
	1b	A	1: 1.2 : 1.2	ACN	5	60 149.29, 149.88
	1b	G	1: 1.2 : 1.2	ACN	24	44 149.29, 149.88
	1b	B	1: 1.2 : 1.2	EtOAc	6	73 149.29, 149.88

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	1b	B*	1: 1.2 : 1.2	EtOAc	7	50	149.29, 149.88
	1b	B	1: 1.2 : 1.2	DCM	1	93	149.29, 149.88
	1c	A	1: 1.2 : 1.2	DCM	3	89	148.39, 149.15
	1c	A	1: 1.2 : 1.2	ACN	20	-	N/A
5	1c	A	1: 1.2 : 1.2	tol	20	-	N/A
	1c	B	1: 1.2 : 1.2	ACN	20	80	148.39, 149.15
	1c	B	1: 1.2 : 1.2	EtOAc	3	66	148.39, 149.15
	1c	B	1: 1.2 : 1.2	DCM	3	74	148.39, 149.15
	1d	D	1: 2.0 : 1	DCM	2	70	149.14, 149.57
10	1d	E	1: 1.4 : 0.3	DCM	2	86	149.14, 149.57
	1d	D	1: 1.1 : 1	DCM	3	94	149.14, 149.57
	1d	O	1: 1.2 : 0.6	DCM	3	41	149.14, 149.57
	1d	A	1: 1.2 : 1.2	DCM	3	86	149.14, 149.57
	1d	B	1: 1.2 : 1.2	DCM	3	88	149.14, 149.57
15	1d	C	1: 1.2 : 1	DCM	3	78	149.14, 149.57
	1d	C	1: 1.2 : 1.2	DCM	3	87	149.14, 149.57
	1d	P	1: 1.2 : 1.2	DCM	3	si.	N/A
	2a	B	1: 1.2 : 1.1	DCM	0.75	75	150.94, 151.67
	3a	B	1: 1.2 : 1.1	DCM	0.75	95	150.60, 151.05
20	4a	B	1: 1.2 : 1.1	DCM	0.8	96	149.66, 151.59
	2b	B	1: 1.2 : 1.2	DCM	2	94	150.77, 151.35
	3b	B	1: 1.2 : 1.2	DCM	2	90	149.85, 150.72
	4f	B	1: 1.2 : 1.2	DCM	2	92	150.76, 150.82
	2c	B	1: 1.2 : 1.2	DCM	2	86	150.71, 150.95
25	3c	B	1: 1.2 : 1.2	DCM	2	82	149.43, 150.37
	4c	B	1: 1.2 : 1.2	DCM	2	94	150.23, 150.82
	2e	B	1: 1.2 : 1.2	DCM	2	88	150.86, 151.39
	3e	B	1: 1.2 : 1.2	DCM	2	84	150.22, 150.61
	4d	B	1: 1.2 : 1.2	DCM	3	95	150.69, 150.83
30	1f	B	1: 1.2 : 1.2	DCM	2	91	149.14, 149.67
	10	Q	1: 1.2 : 1.2	DCM	2	14	149.34, 149.94
	1b	R*	1: 1.2 : 1.2	DCM	2	-	N/A
	1b	S*	1: 1.2 : 1.2	DCM	2	sl.	N/A
	1b	T*	1: 1.2 : 1.2	DCM	2	89	149.29, 149.88
35	1b	U*	1: 1.2 : 1.2	DCM	2	sl.	N/A

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1g	B	1: 1.1 : 1.2	DCM	1	60	149.26, 149.92
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Note: I = compound (nucleoside)
II = activator (Act.)
P[III] = phosphitylating agent
(bisamidite reagent)

- = no reaction

* = *in situ*

Si. = silylation

Sl. = slow reaction

10 Cmpd. = compound, see Figure 1

Act. = activator

Sol. = solvent

DCM = dichloromethane

ACN = acetonitrile

15 EtOAc = ethyl acetate

tol = toluene

Activators A = pyridine hydrochloride

B = Pyridinium trifluoroacetate

20 C = Pyridinium triflate

D = tetrazole

E = diisopropylammonium tetrazolide

F = 4,5-dicyanoimidazole

G = imidazole hydrochloride

25 H = imidazolium triflate

I = aniline hydrochloride

J = *p*-anisidinium trifluoroacetate

K = *p*-toluidine hydrochloride

L = o-toluidine hydrochloride

30 M = 2-amino-4,6-dimethylpyrimidine
trifluoroacetate

N = 1,10-phenanthroline trifluoroacetate

O = chlorotrimethylsilane (TMSCl)

P = 1-(trimethylsilyl)imidazole

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Q = poly(4-vinylpyridine hydrochloride)

R = pyridinium acetate

S = pyridinium chloroacetate

T = pyridinium dichloroacetate

5 U = pyridinium trichloroacetate

Preparation of Intersugar Linkages Using Pyridinium Salt/Substituted Imidazole Actoivators

Example 14

Synthesis of T-T phosphorothioate dimer:

10 100 milligram (4 mmole) of 5'-O-Dimethoxytritylthymidine bonded to CPG (controlled pore glass) through an ester linkage was taken in a glass reactor, and a dichloromethane solution of 2% dichloroacetic acid (volume/volume) was added to deprotect the 5'-hydroxyl
15 group. The product was washed with dichloromethane and then with acetonitrile. Then, a 0.2 M solution of 5'-O-(4,4'-dimethoxytrityl)thymidine-3'-O-(2-cyanoethyl)-N,N-diisopropylphosphoramidite) in acetonitrile and a 0.22 M solution of pyridinium trifluoroacetate and 0.11M solution
20 of 1-methylimidazole in acetonitrile was added, and reacted at room temperature for 5 minutes. The product was washed with acetonitrile, and then a 0.05 M solution of Beaucage reagent in acetonitrile was added and reacted at room temperature for 5 minutes. This sulfurization step was
25 repeated one more time for 5 minutes. The support was washed with acetonitrile and then a solution of acetic anhydride/lutidine/THF (1:1:8), and N-methyl imidazole/THF was added to cap the unreacted 5'-hydroxyl group. The product was washed with acetonitrile.

30 The carrier containing the compound was treated with 30% aqueous ammonium hydroxide solution for 90 minutes. The aqueous solution was filtered, concentrated under reduced pressure to give phosphorothioate dimer of T-T.

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Example 15**Synthesis of C-T phosphorothioate dimer:**

100 milligram (4 mmole) of 5'-O-Dimethoxytritylthymidine bonded to CPG (controlled pore
5 glass) through an ester linkage was taken in a glass
reactor, and a dichloromethane solution of 2% dichloroacetic
acid (volume/volume) was added to deprotect the 5'-hydroxyl
group. The product was washed with acetonitrile. Then, a
0.2 M solution of N⁴-Benzoyl-5'-O-(4,4'-dimethoxytrityl)-2'-
10 deoxycytidine-3'-O-(2-cyanoethyl)-N,N-
diisopropylphosphoramidite) in acetonitrile and a 0.22 M
solution of pyridinium trifluoroacetate and 0.11 M solution
of 1-methylimidazole in acetonitrile was added, and reacted
at room temperature for 5 minutes. The product was washed
15 with acetonitrile, and then a 0.05 M solution of Beaucage
reagent in acetonitrile was added and reacted at room
temperature for 5 minutes. This sulfurization step was
repeated one more time for 5 minutes. The support was washed
with acetonitrile and then a solution of acetic
20 anhydride/lutidine/THF (1:1:8), and N-methyl imidazole/THF
was added to cap the unreacted 5'-hydroxyl group. The
product was washed with acetonitrile.

The carrier containing the compound was treated
with 30% aqueous ammonium hydroxide solution for 90 minutes
25 and then incubated at 55°C for 12 hours. The aqueous solution
was filtered, concentrated under reduced pressure and then
treated at room temperature with 1.0 M solution of tetra-n-
butyl ammonium fluoride in THF to give a phosphorothioate
dimer of dC-T.

30 Example 16**Synthesis of 5'-TTTTTTT-3' phosphorothioate heptamer:**

50 milligram (2 mmole) of 5'-O-dimethoxytritylthymidine bound to CPG (controlled pore
glass) through an ester linkage was taken up in a glass

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reactor, and a toluene solution of 3% dichloroacetic acid (volume/volume) was added to deprotect the 5'-hydroxyl group. The product was washed with acetonitrile. Then, a 0.2 M solution of 5'-O-(4,4'-dimethoxytrityl)thymidine-3'-O-
5 (2-cyanoethyl N,N-diisopropylphosphoramidite) in acetonitrile and a 0.22 M solution of pyridinium trifluoroacetate and 0.11 M solution of 1-methylimidazole in acetonitrile was added, and allowed to react at room temperature for 5 minutes. The product was washed with
10 acetonitrile, and then a 0.2 M solution of phenylacetyl disulfide in acetonitrile:3-picoline (1:1 v/v) was added and allowed to react at room temperature for 3 minutes. This sulfurization step was repeated one more time for 3 minutes. The support was washed with acetonitrile, and then a
15 solution of acetic anhydride/lutidine/THF (1:1:8), and N-methyl imidazole/THF was added to cap any unreacted 5'-hydroxyl group. The product was washed with acetonitrile.

This complete cycle was repeated five more times to produce the completely protected thymidine heptamer. The
20 carrier containing the compound was treated with 30% aqueous ammonium hydroxide solution for 90 minutes at room temperature. The aqueous solution was filtered, and concentrated under reduced pressure to give a phosphorothioate heptamer, TTTTTTT.

25 Example 17

Synthesis of 5'-d(GACT)-3' phosphorothioate tetramer:

50 milligram (2 mmole) of 5'-O-dimethoxytritylthymidine bound to CPG (controlled pore glass) through an ester linkage was taken up in a glass
30 reactor, and a toluene solution of 3% dichloroacetic acid in toluene (volume/volume) was added to deprotect the 5'-hydroxyl group. The product was washed with acetonitrile. Then, a 0.2 M solution of 5'-O-(4,4'-dimethoxytrityl)thymidine-3'-O-(2-cyanoethyl N,N-

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diisopropylphosphoramidite) in acetonitrile and a 0.22 M solution of pyridinium trifluoroacetate and 0.11 M solution of 1-methylimidazole in acetonitrile was added, and allowed to react at room temperature for 5 minutes. The product was
5 washed with acetonitrile, and then a 0.2 M solution of phenylacetyl disulfide in acetonitrile:3-picoline (1:1 v/v) was added and allowed to react at room temperature for 3 minutes. This sulfurization step was repeated one more time for 3 minutes. The support was washed with acetonitrile and
10 then a solution of acetic anhydride/lutidine/THF (1:1:8), and N-methyl imidazole/THF was added to cap the unreacted 5'-hydroxyl group. The product was washed with acetonitrile.

A solution of 3% dichloroacetic acid in toluene (volume/volume) was added to deprotect the 5'-hydroxyl
15 group. The product was washed with acetonitrile. Then, a 0.2 M solution of N⁴-benzoyl-5'-O-(4,4'-dimethoxytrityl)-2'-deoxycytidine-3'-O-(2-cyanoethyl N,N-diisopropylphosphoramidite) in acetonitrile and a 0.22 M solution of pyridinium trifluoroacetate and 0.11 M solution
20 of 1-methylimidazole in acetonitrile was added, and allowed to react at room temperature for 5 minutes. The product was washed with acetonitrile, and then a 0.2 M solution of phenylacetyl disulfide in acetonitrile:3-picoline (1:1 v/v) was added and allowed to react at room temperature for 3
25 minutes. This sulfurization step was repeated one more time for 3 minutes. The support was washed with acetonitrile and then a solution of acetic anhydride/lutidine/THF (1:1:8), and N-methyl imidazole/THF was added to cap any unreacted 5'-hydroxyl group. The product was washed with acetonitrile.

30 A solution of 3% dichloroacetic acid in toluene (volume/volume) was added to deprotect the 5'-hydroxyl group. The product was washed with acetonitrile. Then, a 0.2 M solution of N⁶-benzoyl-5'-O-(4,4'-dimethoxytrityl)-2'-deoxyadenosine-3'-O-(2-cyanoethyl N,N-diisopropylphosphoramidite) in anhydrous acetonitrile and a
35

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0.22 M solution of pyridinium trifluoroacetate and 0.11 M solution of 1-methylimidazole in acetonitrile was added, and allowed to react at room temperature for 5 minutes. The product was washed with acetonitrile, and then a 0.2 M solution of phenylacetyl disulfide in acetonitrile:3-picoline (1:1 v/v) was added and allowed to react at room temperature for 3 minutes. This sulfurization step was repeated one more time for 3 minutes. The support was washed with acetonitrile and then a solution of acetic anhydride/lutidine/THF (1:1:8), and N-methyl imidazole/THF was added to cap the unreacted 5'-hydroxyl group. The product was washed with acetonitrile.

A solution of 3% dichloroacetic acid in toluene (volume/volume) was added to deprotect the 5'-hydroxyl group. The product was washed with acetonitrile. Then, a 0.2 M solution of N²-isobutyryl-5'-O-(4,4'-dimethoxytrityl)-2'-deoxyguanosine-3'-O-(2-cyanoethyl N,N-diisopropylphosphoramidite) in acetonitrile and a 0.22 M solution of pyridinium trifluoroacetate and 0.11 M solution of 1-methylimidazole in acetonitrile was added, and allowed to react at room temperature for 5 minutes. The product was washed with acetonitrile, and then a 0.2 M solution of phenylacetyl disulfide in acetonitrile:3-picoline (1:1 v/v) was added and allowed to react at room temperature for 3 minutes. This sulfurization step was repeated one more time for 3 minutes. The support was washed with acetonitrile and then a solution of acetic anhydride/lutidine/THF (1:1:8), and N-methyl imidazole/THF was added to cap any unreacted 5'-hydroxyl group. The product was washed with acetonitrile.

The carrier containing the compound was treated with 30% aqueous ammonium hydroxide solution for 90 minutes at room temperature and then incubated at 55° C for 24 hour. The aqueous solution was filtered, concentrated under reduced pressure to give a phosphorothioate tetramer of 5'-dG-dA-dC-T-3'.

Example 18

Synthesis of fully-modified 5'-d(TCC-CGC-CTG-TGA-CAT-GCA-TT)-3' phosphorothioate 20-mer

The synthesis of the above sequence was performed on a
5 Pharmacia OligoPilot II Synthesizer on a 180 mmole scale
using the cyanoethyl phosphoramidites and Pharmacia's primar
support. Detritylation was performed using 3%
dichloroacetic acid in toluene (volume/volume). Activation
of phosphoramidites was done with a 0.22 M solution of
10 pyridinium trifluoroacetate and 0.11 M solution of 1-
methylimidazole. Sulfurization was performed using a 0.2 M
solution of phenylacetyl disulfide in acetonitrile:3-
picoline (1:1 v/v) for 2 minutes. At the end of synthesis,
the support was washed with acetonitrile, cleaved,
15 deprotected and purified in the usual manner.

Example 19

Synthesis of fully-modified 5'-d(GCC-CAA-GCT-GGC-ATC-CGT-CA)-3' phosphorothioate 20-mer

The synthesis of the above sequence was performed on a
20 Pharmacia OligoPilot II Synthesizer on a 180 μ mole scale
using the cyanoethyl phosphoramidites and Pharmacia's primar
support. Detritylation was performed using 3% dichloroacetic
acid in toluene (volume/volume). Activation of
phosphoramidites was done with a 0.22 M solution of
25 pyridinium trifluoroacetate and 0.11 M solution of 1-
methylimidazole. Sulfurization was performed using a 0.2 M
solution of phenylacetyl disulfide in acetonitrile:3-
picoline (1:1 v/v) for 2 minutes. At the end of synthesis,
the support was washed with acetonitrile, cleaved,
30 deprotected and purified in the usual manner.

Example 20

Synthesis of fully-modified 5'-d(GCG-TTT-GCT-CTT-CTT-CTT-GCG)-3' phosphorothioate 21-mer

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The synthesis of the above sequence was performed on a Pharmacia OligoPilot II Synthesizer on a 180 μ mole scale using the cyanoethyl phosphoramidites and Pharmacia's primar support. Detritylation was performed using 3% dichloroacetic acid in toluene (volume/volume). Activation of phosphoramidites was done with a 0.22 M solution of pyridinium trifluoroacetate and 0.11 M solution of 1-methylimidazole. Sulfurization was performed using a 0.2 M solution of phenylacetyl disulfide in acetonitrile:3-picoline (1:1 v/v) for 2 minutes. At the end of synthesis, the support was washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

Example 21

Synthesis of fully-modified 5'-d(GTT-CTC-GCT-GGT-GAG-TTT-CA)-3' phosphorothioate 20-mer

The synthesis of the above sequence was performed on a Pharmacia OligoPilot II Synthesizer on a 180 μ mole scale using the cyanoethyl phosphoramidites and Pharmacia's primar support. Detritylation was performed using 3% dichloroacetic acid in toluene (volume/volume). Activation of phosphoramidites was done with a 0.22 M solution of pyridinium trifluoroacetate and 0.11 M solution of 1-methylimidazole. Sulfurization was performed using a 0.2 M solution of phenylacetyl disulfide in acetonitrile:3-picoline (1:1 v/v) for 2 minutes. At the end of synthesis, the support was washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

Example 22

Synthesis of fully-modified 5'-d(TCC-GTC-ATC-GCT-CCT-CAG-GG)-3' phosphorothioate 20-mer

The synthesis of the above sequence was performed on a Pharmacia OligoPilot II Synthesizer on a 180 μ mole scale

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using the cyanoethyl phosphoramidites and Pharmacia's primar support. Detritylation was performed using 3% dichloroacetic acid in toluene (volume/volume). Activation of phosphoramidites was done with a 0.22 M solution of pyridinium trifluoroacetate and 0.11 M solution of 1-methylimidazole. Sulfurization was performed using a 0.2 M solution of phenylacetyl disulfide in acetonitrile:3-picoline (1:1 v/v) for 2 minutes. At the end of synthesis, the support was washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

Example 23

Synthesis of fully-modified 5'-d(TCC-CGC-CTG-TGA)-2'-methoxyethyl-(CAT-GCA-TT)-3' phosphorothioate 20-mer

The synthesis of the above sequence was performed on a Milligen 8800 Synthesizer on a 282 mmole scale using the cyanoethyl phosphoramidites and Pharmacia's primar support. Detritylation was performed using 3% dichloroacetic acid in toluene (volume/volume). Activation of phosphoramidites was done with a 0.22 M solution of pyridinium trifluoroacetate and 0.11 M solution of 1-methylimidazole. Sulfurization was performed using a 0.4 M solution of phenylacetyl disulfide in acetonitrile:3-picoline (1:1 v/v) for 6 minutes. At the end of synthesis, the support was washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

Example 24

Synthesis of fully-modified 5'-d(TCC-CGC-CTG-TGA)-2'-methoxyethyl-(CAT-GCA-TT)-3' phosphorothioate 20-mer

The synthesis of the above sequence was performed on a Pharmacia OligoPilot II Synthesizer on a 250 mmole scale using the cyanoethyl phosphoramidites and Pharmacia's primar support. Detritylation was performed using 3% dichloroacetic acid in toluene (volume/volume). Activation of phosphoramidites was done with a 0.22 M solution of

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pyridinium trifluoroacetate and 0.11 M solution of 1-methylimidazole. Sulfurization was performed using a 0.4 M solution of phenylacetyl disulfide in acetonitrile:3-picoline (1:1 v/v) for 6 minutes. At the end of synthesis, the support was washed with acetonitrile, cleaved, deprotected and purified in the usual manner

Example 25

Synthesis of fully-modified 5'-d(GC^{me}C^{me}-C^{me}AA-GC^{me}T-GGC^{me})-2'-methoxyethyl-(AU^{me}C^{me}-C^{me}GU^{me}-C^{me}A)-3' phosphorothioate 20-mer

10 The synthesis of the above sequence was performed on a OligoPilot II on a 200 mmole scale using the cyanoethyl phosphoramidites and Pharmacia's primar support. Detritylation was performed using 3% dichloroacetic acid in toluene (volume/volume). Activation of phosphoramidites was
15 done with a 0.22 M solution of pyridinium trifluoroacetate and 0.11 M solution of 1-methylimidazole. Sulfurization was performed using a 0.2 M solution of phenylacetyl disulfide in acetonitrile:3-picoline (1:1 v/v) for 3 minutes. At the end of synthesis, the support was washed with acetonitrile,
20 cleaved, deprotected and purified in the usual manner.

Example 26

Synthesis of fully-modified 5'-d(TGG-TGG_TGG_TGG_TGG_TGG-T)-3' phosphorothioate 20-mer

25 In order to compare the extent of formation of (n+1)-mers during the oligonucleotide synthesis between the two activators, the following experiment was conducted:

The synthesis of the above sequence was performed on a OligoPilot I on a 30 mmole scale using the cyanoethyl phosphoramidites and Pharmacia's primar support.
30 Detritylation was performed using 3% dichloroacetic acid in toluene (volume/volume). Activation of phosphoramidites was done with a 0.22 M solution of pyridinium trifluoroacetate and 0.11 M solution of 1-methylimidazole. Sulfurization was

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performed using a 0.2 M solution of phenylacetyl disulfide in acetonitrile:3-picoline (1:1 v/v) for 3 minutes. At the end of synthesis, the support was washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

- 5 The above synthesis was repeated with 0.45 M solution of 1H-tetrazole. At the end of synthesis, the support was washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

10 The oligonucleotides were analyzed by capillary gel electrophoresis. A comparison of the two electropherograms shows that the two activators perform at the same efficiency.

Preparation of Internucleotide Linkages Between 2'-substituted Nucleosides Using Imidazolium Triflate Activator

15 **Example 27**

Synthesis of pyridinium tetrafluoroborate, pyrinium hexafluorophosphate, imidazolium salt and benzimidazolium salt

20 Pyridinium tetrafluoroborate is prepared according to the procedure described by Brill et al., *J. Am. Chem. Soc.*, 1991 113, 3972.

Pyridinium tetrafluoroborate is ion-exchanged with sodium hexafluorophosphate to give pyridinium hexafluorophosphate.

25 Imidazolium triflate is prepared according to the procedure of Kataoka et al., *Nucleic Acids Symposium Series*, 1998, 37, 21-22).

Benzimidazolium triflate is synthesized according to the reported procedure of Hayakawa et al., *J. Org. Chem.*, 1996, 61, 7996-7997.

Example 28

30 **Synthesis of benzimidazolium tetrafluoroborate**

To a solution of benzimidazole (10g, 84.6 mmol) in dichloromethane (30 mL) is added dropwise tetrafluoroboric

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acid as its etherate (85%, HBF_4 by volume, Aldrich Chemicals Co.) with stirring at 0°C . The reaction mixture is diluted with diethylether (100 mL) to precipitate the title compound. The title compound is filtered, washed with ether and
5 recrystallized from ether.

Example 29**Synthesis of imidazolium tetrafluoroborate**

To a solution of imidazole (20 mmol) in dichloromethane (30 mL) at 0°C HBF_4 (20 mmol, 3.8g of a diethyl etherate) in
10 dichloromethane is added dropwise. The reaction mixture is diluted with diethyl ether (100 mL) to precipitate the title compound. It is then filtered, washed with ether and recrystallized from ether.

15 Example 30**Synthesis of imidazolium hexafluorophosphate**

Hexafluorophosphoric acid (65% in water) is purchased from Fluka and evaporated with pyridine three times to concentrate. A solution of imidazole or benzimidazole (20
20 mmol) in ether (100 mL) is treated with 20 mmol of evaporated hexafluorophosphoric acid under stirring and at 0°C . After mixing the solution is evaporated and the slurry is treated with anhydrous ether. The salt is isolated by filtration, followed by washing with ether and drying in vacuo.

25 Example 31**Synthesis of nucleobase-protected amidite monomer units derived from 2'-MOE nucleoside precursors using imidazolium salts**

The nucleosidic monomers having 2'-O-(methoxyethyl)
30 modification are treated with 2-cyanoethyl-N,N,N',N'-tetraisopropyl phosphorodiamidite (1.2 equivalents) and imidazolium salt or benzimidazolium salt (0.5 equivalent) in dry methylenechloride at ambient temperature for about 30-60

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minutes. Reaction progress and formation of the respective amidite is monitored by tlc. This general procedure is used to convert selected 2'-O-methoxyethoxy (2'-O-MOE) protected nucleosides into the respective phosphoramidites. One
5 equivalent of selected nucleosides 2'-O-(MOE)-5'-O-DMT-6-N-benzoyl-adenosine, 2'-O-(MOE)-5'-O-DMT-4-N-benzoyl-cytidine, 2'-O-(MOE)-5'-O-DMT-N-2-isobutyryl-guanosine, 2'-O-(MOE)-5'-O-DMT-5-methyluridine, 2'-O-(MOE)-5'-O-DMT-uridine, 2'-O-(MOE)-5'-O-DMT-5-methyl-4-N-benzoyl-cytidine in anhydrous
10 dichloromethane is treated with 2-cyanoethyl-N,N,N',N'-tetraisopropyl phosphorodiamidite (1.2 equivalents) and either imidazolium salt or benzimidazolium salt (0.5 equivalent) at ambient temperature for 30-60 minutes under argon. The reaction mixture is directly loaded onto a silica
15 gel column and the product eluted with a gradient of ethylacetate/hexane. Desired product for each respective amidite is identified by tlc and collected and concentrated. Purity is determined by ¹H and ³¹P NMR studies.

Example 32

20 Synthesis of nucleobase-unprotected amidite monomers derived from 2'-MOE nucleoside precursors using imidazolium salts

The nucleoside monomers without the protecting group for exocyclic amines are synthesized in a similar manner to the previous example. 2'-O-(MOE)-5'-O-DMT adenosine, 2'-O-(MOE)-
25 5'-O-DMT-cytidine, 2'-O-(MOE)-5'-O-guanosine, 2'-O-(MOE)-5'-O-DMT-5-methyluridine, 2'-O-(MOE)-5'-O-DMT-uridine, 2'-O-(MOE)-5'-O-DMT-2-amino-adenosine, 2'-O-(MOE)-5'-O-DMT-5-methylcytidine, in each case 1 equivalent, is taken in anhydrous methylenechloride/DMF mixture and treated with 2-
30 cyanoethyl-N,N,N',N'-tetraisopropyl phosphorodiamidite (1.5 equivalents) and one of the imidazolium salts or benzimidazolium salts (0.5 equivalents) at ambient temperature for 30-60 mins under argon. The reaction mixture

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is evaporated, toluene is added and reevaporated and the residue is dissolved in anhydrous methylenechloride and eluted with ethylacetate solvent. The phosphoramidite products are pooled and characterized by ^{31}P NMR.

5 Example 33

Synthesis of fully-modified 5'-d(TCC-CGC-CTG-TGA-CAT-GCA-TT) - 3' phosphorothioate 20-mer

The synthesis of the above sequence is performed on an Expedite (Millipore) Synthesizer on a 1 micromole scale using 2-cyanoethyl phosphoramidites and CPG support. Detritylation is performed using 3% dichloroacetic acid in methylene chloride. Activation of phosphoramidites is done with a 0.22 M solution of pyridinium tetrafluoroborate and 0.11 M solution of 1-methylimidazole. Sulfurization is performed using Beaucage reagent. At the end of synthesis, the support is washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

Example 34

Synthesis of fully-modified 5'-d(GCC-CAA-GCT-GGC-ATC-CGT-CA) - 3' phosphorothioate 20-mer

The above sequence is prepared using an Expedite (Millipore) Synthesizer on a 1 micromole scale using 2-cyanoethyl phosphoramidites and CPG support. Detritylation is performed using 3% dichloroacetic acid in methylene chloride. Phosphoramidites are activated with a 0.22 M solution of pyridinium tetrafluoroborate and 0.11 M solution of 1-methylimidazole. Sulfurization is performed using Beaucage reagent. At the end of synthesis, the support is washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

Example 35

Synthesis of fully-modified 5'-d(GCG-TTT-GCT-CTT-CTT-CTT-

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GCG)-3' phosphorothioate 21-mer

The above sequence is prepared on an Expedite (Millipore) Synthesizer on a 1 micromole scale using the 2-cyanoethyl phosphoramidites and CPG support. Detritylation is performed using 3% dichloroacetic acid in methylene chloride. Phosphoramidites are activated with a 0.22 M solution of pyridinium tetrafluoroborate and 0.11 M solution of 1-methylimidazole. Sulfurization is performed using Beaucage reagent. After synthesis, the support is washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

Example 36**Synthesis of fully-modified 5'-d(GTT-CTC-GCT-GGT-GAG-TTT-CA)-3' phosphorothioate 20-mer**

The above sequence is prepared on an Expedite (Millipore) Synthesizer on a 1 micromole scale using the 2-cyanoethyl phosphoramidites and CPG support. Detritylation is performed using 3% dichloroacetic acid in methylene chloride. Phosphoramidites are activated with a 0.22 M solution of pyridinium tetrafluoroborate and 0.11 M solution of 1-methylimidazole. Sulfurization is performed using Beaucage reagent. At the end of synthesis, the support is washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

Example 37**Synthesis of fully-modified 5'-d(TCC-GTC-ATC-GCT-CCT-CAG-GG)-3' phosphorothioate 20-mer**

The above sequence is prepared on an Expedite (Millipore) Synthesizer on a 1 micromole scale using the 2-cyanoethyl phosphoramidites and CPG support. Detritylation is performed using 3% dichloroacetic acid in methylene chloride. Phosphoramidites are activated with a 0.22 M solution of pyridinium tetrafluoroborate and 0.11 M solution

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of 1-methylimidazole. Sulfurization is performed using Beaucage reagent. At the end of synthesis, the support is washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

5 Example 38

Synthesis of fully-modified 5'-d(TCC-CGC-CTG-TGA)-2'-O-(MOE)-(CAT-GCA-TT)-3' phosphorothioate 20-mer

The above sequence is prepared on a Millipore Expedite Synthesizer on a 1 micromole scale using the 2-cyanoethyl phosphoramidites and CPG support. Detritylation is performed using 3% dichloroacetic acid in methylene chloride. Phosphoramidites are activated with a 0.22 M solution of pyridinium tetrafluoroborate and 0.11 M solution of 1-methylimidazole. Sulfurization is performed using Beaucage reagent. At the end of synthesis, the support is washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

Example 39

Synthesis of fully-modified 5'-d(GCC CAA GCT GGC)-2'-O-(MOE)-(ATC CCG TCA)-3' phosphorothioate 20-mer

The above sequence is prepared on an Expedite (Millipore) Synthesizer on a 1 micromole scale using the 2-cyanoethyl phosphoramidites and CPG support. Detritylation is performed using 3% dichloroacetic acid in methylene chloride. Phosphoramidites are activated with a 0.22 M solution of pyridinium tetrafluoroborate and 0.11 M solution of 1-methylimidazole. Sulfurization is performed using Beaucage reagent. At the end of synthesis, the support is washed with acetonitrile, cleaved, deprotected and purified in the usual manner

Example 40

Synthesis of fully-modified 5'-d(GC^{me}C^{me}-C^{me}AA-GC^{me}T-GGC^{me})-2'-O-

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(MOE) - (AU^{me}C^{me}-C^{me}GU^{me}-C^{me}A) -3' phosphorothioate 20-mer

The above sequence is prepared on an Expedite (Millipore) Synthesizer on a 1 micromole scale using the 2-cyanoethyl phosphoramidites and CPG support. Detritylation
5 is performed using 3% dichloroacetic acid in methylene chloride. Phosphoramidites are activated with a 0.22 M solution of pyridinium tetrafluoroborate and 0.11 M solution of 1-methylimidazole. Beaucage reagent is used for phosphorothioate synthesis. At the end of synthesis, the
10 support is washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

Example 41**Synthesis of 2'-O-MOE gapmers**

Stock solutions of 2'-O-MOE amidites (0.1 M) are made in
15 anhydrous acetonitrile and loaded onto an Expedite Nucleic Acid synthesis system (Millipore) to prepare oligonucleotides. Commercially available deoxyamidites (A, T, C and G, PerSeptive Biosystem) are also made into stock solutions (0.1 M) with anhydrous acetonitrile. All syntheses
20 are carried out in the DMT ON mode. For the coupling of the 2'-O-MOE amidites coupling time is extended to 10 minutes and this step is carried out twice. All other steps in the protocol supplied by Millipore are used except the extended coupling time (240 seconds). Activation of phosphoramidites
25 is done with a 0.22 M solution of pyridinium tetrafluoroborate and 0.11 M solution of 1-methylimidazole. Beaucage reagent is used for phosphorothioate synthesis. The overall coupling efficiencies are expected to be greater than 95%. The oligonucleotides are cleaved from the controlled
30 pore glass (CPG) supports and deprotected under standard conditions using concentrated aqueous NH₄OH (30%) at 55°C. 5'-O-DMT containing oligomers are then purified by reverse phase liquid chromatography (C-4, Waters, 7-8 x 300 mm, A=50

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mM triethylammonium acetate pH 1, B=100%CH₃CN, 5 to 60% B in 60 minutes). Detritylation with aqueous 80% acetic acid (1 mL, 30 min., room temperature), evaporation, followed by desalting by using Sephadese G-25 column will yield the oligonucleotides expectedly as foams. All oligomers are analyzed by CGE, HPLC and mass spectrometry.

2'-MOE GAPMERS

	Sequence 5'-3'	Backbone	Target
10	T*sT*sC*sT*sC*s GsCsCsCsGsCsTsCs C*sT*sC*sC*sT*sC*sC*	P=S	c-raf
	T*sT*sC*sT*sC*s GsCsTsGsGsTsGs AsGs T*sT*sT*sC*sA*	P=S	pkc-a
	T*oT*oC*oT*oC*s GsCsCsCsGsCsTsCs C*oT*oC*oC*oT*oC*oC*	P=O, P=S, P=O	c-raf
15	T*oT*oC*oT*oC*s GsCsTsGsGsTsGs AsGs T*oT*oT*oC*oA*	P=O, P=S,	pkc-a

* = 2'-O-MOE

C's are all 5-methyl substituted

s = phosphorothioate internucleotide linkages

20 o = phosphodiester internucleotide linkages

Example 42

Synthesis of uniformly modified 2'-modified oligonucleotide

2-O-MOE amidites of A, ^{5me}C, G and T are dissolved in anhydrous acetonitrile to give 0.1 M solution. These solutions are loaded onto an Expedite Nucleic Acid Synthesis system (Millipore) to synthesize the oligonucleotides. Activation of phosphoramidites is done with a 0.22 M solution of pyridinium tetrafluoroborate and 0.11 M solution of 1-methylimidazole. The coupling efficiencies are expected to be more than 90 %. All steps in the protocol supplied by Millipore are used except the activation step. Beaucage reagent (0.1 M in acetonitrile) is used as a sulfurizing agent. For diester synthesis, t-BuOOH is used as the oxidizing agent.

35 The oligomers are cleaved from the controlled pore glass (CPG) supports and deprotected under standard conditions

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using concentrated aqueous NH_4OH (30%) at 55 °C. 5'-O-DMT containing oligomers are then purified by reverse phase high performance liquid chromatography (C-4, Waters, 7.8 x 300 mm, A = 50 mM triethylammonium acetate, pH -7, B = acetonitrile, 5 5-60% of B in 60 min., flow 1.5 mL/minute). Detritylation with aqueous 80% acetic acid and evaporation, followed by desalting in a Sephadex G-25 column will give the oligonucleotides. Oligonucleotides are analyzed by HPLC, CGE and Mass spectrometry.

10

Sequence	Target
5' T*sC*sT*s G*sA*sG*s T*sA*sG*s C*sA*sG*s A*sG*sG*s A*sG*sC*s T*sC* 3'	I C A M , P=S
5' T*C*T*G*A*G*T*A*G*C*A*G*A*G*G*A*G*C*T*C* 3'	I C A M , P=O

15 T* = 2'-O-MOE T, A* = 2'-O-MOE A, C* = 2'-O-MOE ^{5me}C, G* = 2'-O-MOE G

Examples 43-60 Oligonucleotide synthesis employing pyridinium hexafluorophosphate

Example 43

20 **Synthesis of fully-modified 5'-d(TCC-CGC-CTG-TGA-CAT-GCA-TT)-3' phosphorothioate 20-mer**

The synthesis of the above sequence is performed on an Expedite (Millipore) Synthesizer on a 1 micromole scale using the 2-cyanoethyl phosphoramidites and CPG support.

25 Detritylation is performed using 3% dichloroacetic acid in methylene chloride. Activation of phosphoramidites is done with a 0.22 M solution of pyridinium hexafluorophosphate and 0.11 M solution of 1-methylimidazole. Sulfurization is performed using Beaucage reagent. At the end of synthesis,
30 the support is washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

Example 44

Synthesis of fully-modified 5'-d(GCC-CAA-GCT-GGC-ATC-CGT-CA)-

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3' phosphorothioate 20-mer

The synthesis of the above sequence is performed on an Expedite (Millipore) Synthesizer on a 1 micromole scale using the 2-cyanoethyl phosphoramidites and CPG support.

- 5 Detritylation is performed using 3% dichloroacetic acid in methylene chloride. Activation of phosphoramidites is done with a 0.22 M solution of pyridinium hexafluorophosphate and 0.11 M solution of 1-methylimidazole. Sulfurization is performed using Beaucage reagent. At the end of synthesis,
10 the support is washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

Example 45**Synthesis of fully-modified 5'-d(GCG-TTT-GCT-CTT-CTT-CTT-GCG)-3' phosphorothioate 21-mer**

- 15 The synthesis of the above sequence is performed on an Expedite (Millipore) Synthesizer on a 1 micromole scale using the 2-cyanoethyl phosphoramidites and CPG support. Detritylation is performed using 3% dichloroacetic acid in methylene chloride. Activation of phosphoramidites is done
20 with a 0.22 M solution of pyridinium hexafluorophosphate and 0.11 M solution of 1-methylimidazole. Sulfurization is performed using Beaucage reagent. At the end of synthesis, the support is washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

25 Example 46**Synthesis of fully-modified 5'-d(GTT-CTC-GCT-GGT-GAG-TTT-CA)-3' phosphorothioate 20-mer**

- The synthesis of the above sequence is performed on an Expedite (Millipore) Synthesizer on a 1 micromole scale using
30 the 2-cyanoethyl phosphoramidites and CPG support. Detritylation is performed using 3% dichloroacetic acid in methylene chloride. Activation of phosphoramidites is done with a 0.22 M solution of pyridinium hexafluorophosphate and

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0.11 M solution of 1-methylimidazole. Sulfurization is performed using Beaucage reagent. At the end of synthesis, the support is washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

5 Example 47

Synthesis of fully-modified 5'-d(TCC-GTC-ATC-GCT-CCT-CAG-GG)-3' phosphorothioate 20-mer

The synthesis of the above sequence is performed on an Expedite (Millipore) Synthesizer on a 1 micromole scale using
10 the 2-cyanoethyl phosphoramidites and CPG support. Detritylation is performed using 3% dichloroacetic acid in methylene chloride. Activation of phosphoramidites is done with a 0.22 M solution of pyridinium hexafluorophosphate and 0.11 M solution of 1-methylimidazole. Sulfurization is
15 performed using Beaucage reagent. At the end of synthesis, the support is washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

Example 48

**Synthesis of fully-modified 5'-d(TCC-CGC-CTG-TGA)-2'-O-(MOE)-
20 (CAT-GCA-TT)-3' phosphorothioate 20-mer**

The synthesis of the above sequence is performed on a Millipore Expedite Synthesizer on a 1 micromole scale using the 2-cyanoethyl phosphoramidites and CPG support. Detritylation is performed using 3% dichloroacetic acid in
25 methylene chloride. Activation of phosphoramidites is done with a 0.22 M solution of pyridinium hexafluorophosphate and 0.11 M solution of 1-methylimidazole. Sulfurization is performed using Beaucage reagent. At the end of synthesis, the support is washed with acetonitrile, cleaved, deprotected
30 and purified in the usual manner.

Example 49

Synthesis of fully-modified 5'-d(GCC CAA GCT GGC)-2'-O-(MOE)-

- 96 -

(ATC CCG TCA)-3' phosphorothioate 20-mer

The synthesis of the above sequence is performed on an Expedite (Millipore) Synthesizer on a 1 micromole scale using the 2-cyanoethyl phosphoramidites and CPG support.

- 5 Detritylation is performed using 3% dichloroacetic acid in methylene chloride. Activation of phosphoramidites is done with a 0.22 M solution of pyridinium hexafluorophosphate and 0.11 M solution of 1-methylimidazole. Sulfurization is performed using Beaucage reagent. At the end of synthesis,
- 10 the support is washed with acetonitrile, cleaved, deprotected and purified in the usual manner

Example 50**Synthesis of fully-modified 5'-d(GC^{me}C^{me}-C^{me}AA-GC^{me}T-GGC^{me})-2'-O-(MOE)-(AU^{me}C^{me}-C^{me}GU^{me}-C^{me}A)-3'-phosphorothioate 20-mer**

- 15 The synthesis of the above sequence is performed on an Expedite (Millipore) Synthesizer on a 1 micromole scale using the 2-cyanoethyl phosphoramidites and CPG support. Detritylation is performed using 3% dichloroacetic acid in methylene chloride. Activation of phosphoramidites is done
- 20 with a 0.22 M solution of pyridinium hexafluorophosphate and 0.11 M solution of 1-methylimidazole. Beaucage reagent is used for phosphorothioate synthesis. At the end of synthesis, the support is washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

25 Example 51**Synthesis of 2'-MOE gapmers**

- A 0.1 M solution of 2'-O-MOE amidites are made in anhydrous acetonitrile and loaded onto an Expedite Nucleic Acid synthesis system (Millipore) to synthesize
- 30 oligonucleotides. All other deoxyamidites (A, T, C and G, PerSeptive Biosystem) used in synthesis are also made as 0.1 M solution in anhydrous acetonitrile. All syntheses are

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carried out in DMT on mode. For the coupling of the 2'-O-MOE
 amidites coupling time is extended to 10 minutes and this
 step is carried out twice. All other steps in the protocol
 supplied by Millipore are used except the extended coupling
 5 time (240 seconds). Activation of phosphoramidites is done
 with a 0.22 M solution of pyridinium hexafluorophosphate and
 0.11 M solution of 1-methylimidazole. Beaucage reagent is
 used for phosphorothioate synthesis. The overall coupling
 efficiencies are expected to be more than 95%. The
 10 oligonucleotides are cleaved from the controlled pore glass
 (CPG) supports and deprotected under standard conditions
 using concentrated aqueous NH_4OH (30%) at 55°C. 5'-O-DMT
 containing oligomers are then purified by reverse phase
 liquid chromatography (C-4, Waters, 7-8 x 300 mm, A=50 mM
 15 triethylammonium acetate pH 1, B=100% CH_3CN , 5 to 60% B in 60
 minutes). Detritylation with aqueous 80% acetic acid (1 mL,
 30 min., room temperature), concentration, followed by
 desalting by using Sephadese G-25 column will give the
 oligonucleotides as a pure foam. All oligomers are then
 20 analyzed by CGE, HPLC and mass spectrometry.

MOE GAPMERS

	Sequence 5'-3'	Backbone	Target
25	T*sT*sC*sT*sC*s GsCsCsCsGsCsTsCs C*sT*sC*sC*sT*sC*sC*	P=S	c-raf
	T*sT*sC*sT*sC*s GsCsTsGsGsTsGs AsGs T*sT*sT*sC*sA*	P=S	pkc-?
	T*oT*oC*oT*oC*s GsCsCsCsGsCsTsCs C*oT*oC*oC*oT*oC*oC*	P=O, P=S, P=O	c-raf
30	T*oT*oC*oT*oC*s GsCsTsGsGsTsGs AsGs T*oT*oT*oC*oA*	P=O, P=S,	pkc-?

*=2'-O-MOE; All C=5-methyl C;

Example 52**General procedure for uniformly modified 2'-modified oligonucleotide synthesis**

2-O-MOE amidites of A, ^{5me}C, G and T are dissolved in anhydrous acetonitrile to give 0.1 M solution. These solutions are loaded onto an Expedite Nucleic Acid Synthesis system (Millipore) to synthesize the oligonucleotides. Activation of phosphoramidites is done with a 0.22 M solution of pyridinium hexafluorophosphate and 0.11 M solution of 1-methylimidazole. The coupling efficiencies are expected to be more than 95%. For the coupling of the first amidite coupling time is extended to 6 minutes and this step is carried out twice. All other steps in the protocol supplied by Millipore are used except the extended coupling time. Beaucage reagent (0.1 M in acetonitrile) is used as a sulfurizing agent. For diester synthesis, t-BuOOH is used as the oxidizing agent. The oligomers are cleaved from the controlled pore glass (CPG) supports and deprotected under standard conditions using concentrated aqueous NH₄OH (30%) at 55 °C. 5'-O-DMT containing oligomers are then purified by reverse phase high performance liquid chromatography (C-4, Waters, 7.8 x 300 mm, A = 50 mM triethylammonium acetate, pH -7, B = acetonitrile, 5-60% of B in 60 min., flow 1.5 mL/minute). Detritylation with aqueous 80% acetic acid and evaporation, followed by desalting in a Sephadex G-25 column will give the oligonucleotides. Oligonucleotides are analyzed by HPLC, CGE and Mass spectrometry.

Sequence	Target
5' T*sC*sT*s G*sA*sG*s T*sA*sG*s C*sA*sG*s A*sG*sG*s A*sG*sC*s T*sC* 3'	I C A M , P=S
5' T*C*T*G*A*G*T*A*G*C*A*G*A*G*G*A*G*C*T*C* 3'	I C A M , P=O

T* = 2'-O-MOE T, A* = 2'-O-MOE A, C* = 2'-O-MOE ^{5me}C, G* = 2'-O-MOE G

EXAMPLES 53-60

Oligonucleotide synthesis using benzimidazolium or imidazolium tetrafluoroborate activator

Example 53

5 **Synthesis of fully-modified 5'-d(TCC-CGC-CTG-TGA-CAT-GCA-TT) - 3' phosphorothioate 20-mer**

The synthesis of the above sequence is performed on an Expedite (Millipore) Synthesizer on a 1 micromole scale using the 2-cyanoethyl phosphoramidites and CPG support.

- 10 Detritylation is performed using 3% dichloroacetic acid in methylene chloride. Activation of phosphoramidites is done with a 0.22 M solution of imidazolium or benzimidazolium tetrafluoroborate and 0.11 M solution of 1-methylimidazole. Sulfurization is performed using Beaucage reagent. At the
- 15 end of synthesis, the support is washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

Example 54

Synthesis of fully-modified 5'-d(GCC-CAA-GCT-GGC-ATC-CGT-CA) - 3'-phosphorothioate 20-mer

- 20 The synthesis of the above sequence is performed on an Expedite (Millipore) Synthesizer on a 1 micromole scale using the 2-cyanoethyl phosphoramidites and CPG support. Detritylation is performed using 3% dichloroacetic acid in methylene chloride. Activation of phosphoramidites is done
- 25 with a 0.22 M solution of imidazolium or benzimidazolium tetrafluoroborate and 0.11 M solution of 1-methylimidazole. Sulfurization is performed using Beaucage reagent. At the end of synthesis, the support is washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

30 **Example 55**

Synthesis of fully-modified 5'-d(GCG-TTT-GCT-CTT-CTT-CTT-

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GCG)-3' phosphorothioate 21-mer

The synthesis of the above sequence is performed on an Expedite (Millipore) Synthesizer on a 1 micromole scale using the 2-cyanoethyl phosphoramidites and CPG support.

- 5 Detritylation is performed using 3% dichloroacetic acid in methylene chloride. Activation of phosphoramidites is done with a 0.22 M solution of imidazolium or benzimidazolium tetrafluoroborate and 0.11 M solution of 1-methylimidazole. Sulfurization is performed using Beaucage reagent. At the
10 end of synthesis, the support is washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

Example 56**Synthesis of fully-modified 5'-d(GTT-CTC-GCT-GGT-GAG-TTT-CA)-3' phosphorothioate 20-mer**

- 15 The synthesis of the above sequence is performed on an Expedite (Millipore) Synthesizer on a 1 micromole scale using the 2-cyanoethyl phosphoramidites and CPG support. Detritylation is performed using 3% dichloroacetic acid in methylene chloride. Activation of phosphoramidites is done
20 with a 0.22 M solution of imidazolium or benzimidazolium tetrafluoroborate and 0.11 M solution of 1-methylimidazole. Sulfurization is performed using Beaucage reagent. At the end of synthesis, the support is washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

25 Example 57**Synthesis of fully-modified 5'-d(GCC CAA GCT GGC)-2'-O-(MOE)-(ATC CCG TCA)-3' phosphorothioate 20-mer**

- The synthesis of the above sequence is performed on an Expedite (Millipore) Synthesizer on a 1 micromole scale using
30 the 2-cyanoethyl phosphoramidites and CPG support. Detritylation is performed using 3% dichloroacetic acid in methylene chloride. Activation of phosphoramidites is done with a 0.22 M solution of imidazolium or benzimidazolium

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tetrafluoroborate and 0.11 M solution of 1-methylimidazole. Sulfurization is performed using Beaucage reagent. At the end of synthesis, the support is washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

5 Example 56

Synthesis of fully-modified 5'-d(TCC-CGC-CTG-TGA)-2'-O-(MOE)-(CAT-GCA-TT)-3' phosphorothioate 20-mer

The synthesis of the above sequence is performed on a Millipore Expedite Synthesizer on a 1 micromole scale using
10 the 2-cyanoethyl phosphoramidites and CPG support. Detritylation is performed using 3% dichloroacetic acid in methylene chloride. Activation of phosphoramidites is done with a 0.22 M solution of imidazolium or benzimidazolium tetrafluoroborate and 0.11 M solution of 1-methylimidazole.
15 Sulfurization is performed using Beaucage reagent. At the end of synthesis, the support is washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

Example 57

Synthesis of fully-modified 5'-d(TCC-CGC-CTG-TGA)-2'-O-(MOE)-(CAT-GCA-TT)-3' phosphorothioate 20-mer
20

The synthesis of the above sequence is performed on an Expedite (Millipore) Synthesizer on a 1 micromole scale using the 2-cyanoethyl phosphoramidites and CPG support. Detritylation is performed using 3% dichloroacetic acid in
25 methylene chloride. Activation of phosphoramidites is done with a 0.22 M solution of imidazolium or benzimidazolium tetrafluoroborate and 0.11 M solution of 1-methylimidazole. Sulfurization is performed using Beaucage reagent. At the end of synthesis, the support is washed with acetonitrile,
30 cleaved, deprotected and purified in the usual manner

Example 58

Synthesis of fully-modified 5'-d(GC^{me}C^{me}-C^{me}AA-GC^{me}T-GGC^{me})-2'-O-

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(MOE) - (AU^{me}C^{me}-C^{me}GU^{me}-C^{me}A) -3' phosphorothioate 20-mer

The synthesis of the above sequence is performed on an Expedite (Millipore) Synthesizer on a 1 micromole scale using the 2-cyanoethyl phosphoramidites and CPG support.

- 5 Detritylation is performed using 3% dichloroacetic acid in methylene chloride. Activation of phosphoramidites is done with a 0.22 M solution of imidazolium or benzimidazolium tetrafluoroborate and 0.11 M solution of 1-methylimidazole. Beaucage reagent is used for phosphorothioate synthesis. At
10 the end of synthesis, the support is washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

Example 59**Synthesis of 2'-MOE gapmers**

- 15 A 0.1 M solution of 2'-O-MOE amidites are prepared in anhydrous acetonitrile and loaded onto an Expedite Nucleic Acid synthesis system (Millipore) to synthesize oligonucleotides. All other deoxyamidites (A, T, C and G, PerSeptive Biosystem) used in synthesis also made as 0.1 M
20 solution in anhydrous acetonitrile. All syntheses are carried out in DMT on mode. For the coupling of the 2'-O-MOE amidites coupling time is extended to 10 minutes and this step is carried out twice. All other steps in the protocol supplied by Millipore are used except the extended coupling
25 time (240 seconds). Activation of phosphoramidites is done with a 0.22 M solution of imidazolium or benzimidazolium tetrafluoroborate and 0.11 M solution of 1-methylimidazole. Beaucage reagent is used for phosphorothioate synthesis. The overall coupling efficiencies are expected to be more than
30 95%. The oligonucleotides are cleaved from the controlled pore glass (CPG) supports and deprotected under standard conditions using concentrated aqueous NH₄OH (30%) at 55°C. 5'-O-DMT containing oligomers are then purified by reverse

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phase liquid chromatography (C-4, Waters, 7-8 x 300 mm, A=50 mM triethylammonium acetate pH 1, B=100%CH₃CN, 5 to 60% B in 60 minutes). Detritylation with aqueous 80% acetic acid (1 mL, 30 min., room temperature), concentration, followed by
 5 desalting by using Sephadese G-25 column will give the oligonucleotides as pure foams. All oligomers are then analyzed by CGE, HPLC and mass spectrometry.

MOE GAPMERS

	Sequence 5'-3'	Backbone	Target
10	T*sT*sC*sT*sC*s GsCsCsCsGsCsTsCs C*sT*sC*sC*sT*sC*sC*	P=S	c-raf
	T*sT*sC*sT*sC*s GsCsTsGsGsTsGs AsGs T*sT*sT*sC*sA*	P=S	pkc-a
15	T*oT*oC*oT*oC*s GsCsCsCsGsCsTsCs C*oT*oC*oC*oT*oC*oC*	P=O, P=S, P=O	c-raf
	T*oT*oC*oT*oC*s GsCsTsGsGsTsGs AsGs T*oT*oT*oC*oA*	P=O, P=S,	pkc-a

20 *≡2'-O-MOE; All C=5-methyl C;

Example 60

Synthesis of uniformly modified 2'-modified oligonucleotide

2-O-MOE amidites of A, ^{5me}C, G and T are dissolved in anhydrous acetonitrile to give 0.1 M solution. These
 25 solutions are loaded onto an Expedite Nucleic Acid Synthesis system (Millipore) to synthesize the oligonucleotides. Activation of phosphoramidites is done with a 0.22 M solution of imidazolium or benzimidazolium tetrafluoroborate and 0.11 M solution of 1-methylimidazole. The coupling efficiencies
 30 are expected to be more than 90%. For the coupling of the first amidite coupling time is extended to 10 minutes and this step is carried out twice. All other steps in the protocol supplied by Millipore are used except the extended coupling time. Beaucage reagent (0.1 M in acetonitrile) is

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used as a sulfurizing agent. For diester synthesis, t-BuOOH is used as the oxidizing agent.

The oligomers are cleaved from the controlled pore glass (CPG) supports and deprotected under standard conditions using

- 5 concentrated aqueous NH_4OH (30%) at 55 °C. 5'-O-DMT containing oligomers are then purified by reverse phase high performance liquid chromatography (C-4, Waters, 7.8 x 300 mm, A = 50 mM triethylammonium acetate, pH -7, B = acetonitrile, 5-60% of B in 60 min., flow 1.5 mL/minute). Detritylation
- 10 with aqueous 80% acetic acid and evaporation, followed by desalting in a Sephadex G-25 column will give the oligonucleotides. Oligonucleotides are analyzed by HPLC, CGE and Mass spectrometry.

Sequence	Target
5' T*sC*sT*s G*sA*sG*s T*sA*sG*s C*sA*sG*s A*sG*sG*s A*sG*sC*s T*sC* 3'	I C A M , P=S
5' T*C*T*G*A*G*T*A*G*C*A*G*A*G*G*A*G*C*T*C* 3'	I C A M , P=O

T* = 2'-O-MOE T, A* = 2'-O-MOE A, C* = 2'-O-MOE ^{5me}C, G* = 2'-

20 O-MOE G

EXAMPLES 61-70

Oligonucleotide Synthesis with imidazolium or benzimidazolium hexafluorophosphate as activator

25

Example 61

Synthesis of fully-modified 5'-d(TCC-CGC-CTG-TGA-CAT-GCA-TT) -
3' phosphorothioate 20-mer

- The synthesis of the above sequence is performed on an
- 30 Expedite (Millipore) Synthesizer on a 1 micromole scale using the 2-cyanoethyl phosphoramidites and CPG support. Detritylation is performed using 3% dichloroacetic acid in methylene chloride. Activation of phosphoramidites is done with a 0.22 M solution of imidazolium or benzimidazolium

hexafluorophosphate and 0.11 M solution of 1-methylimidazole. Sulfurization is performed using Beaucage reagent. At the end of synthesis, the support is washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

5 Example 62

Synthesis of fully-modified 5'-d(GCC-CAA-GCT-GGC-ATC-CGT-CA) - 3' phosphorothioate 20-mer

The synthesis of the above sequence is performed on an Expedite (Millipore) Synthesizer on a 1 micromole scale using
10 the 2-cyanoethyl phosphoramidites and CPG support. Detritylation is performed using 3% dichloroacetic acid in methylene chloride. Activation of phosphoramidites is done with a 0.22 M solution of imidazolium or benzimidazolium hexafluorophosphate and 0.11 M solution of 1-methylimidazole.
15 Sulfurization is performed using Beaucage reagent. At the end of synthesis, the support is washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

Example 63

20 Synthesis of fully-modified 5'-d(GCG-TTT-GCT-CTT-CTT-CTT-GCG) - 3' phosphorothioate 21-mer

The synthesis of the above sequence is performed on an Expedite (Millipore) Synthesizer on a 1 micromole scale using the 2-cyanoethyl phosphoramidites and CPG support. Detritylation is performed using 3% dichloroacetic acid in
25 methylene chloride. Activation of phosphoramidites is done with a 0.22 M solution of imidazolium or benzimidazolium hexafluorophosphate and 0.11 M solution of 1-methylimidazole. Sulfurization is performed using Beaucage reagent. At the end of synthesis, the support is washed with acetonitrile,
30 cleaved, deprotected and purified in the usual manner.

Example 64

Synthesis of fully-modified 5'-d(GTT-CTC-GCT-GGT-GAG-TTT-CA) -

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3' phosphorothioate 20-mer

The synthesis of the above sequence is performed on an Expedite (Millipore) Synthesizer on a 1 micromole scale using the 2-cyanoethyl phosphoramidites and CPG support.

- 5 Detritylation is performed using 3% dichloroacetic acid in methylene chloride. Activation of phosphoramidites is done with a 0.22 M solution of imidazolium or benzimidazolium hexafluorophosphate and 0.11 M solution of 1-methylimidazole. Sulfurization is performed using Beaucage reagent. At the
- 10 end of synthesis, the support is washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

Example 65**Synthesis of fully-modified 5'-d(TCC-GTC-ATC-GCT-CCT-CAG-GG) - 3' phosphorothioate 20-mer**

- 15 The synthesis of the above sequence is performed on an Expedite (Millipore) Synthesizer on a 1 micromole scale using the 2-cyanoethyl phosphoramidites and CPG support. Detritylation is performed using 3% dichloroacetic acid in methylene chloride. Activation of phosphoramidites is done
- 20 with a 0.22 M solution of imidazolium or benzimidazolium hexafluorophosphate and 0.11 M solution of 1-methylimidazole. Sulfurization is performed using Beaucage reagent. At the end of synthesis, the support is washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

25 Example 66**Synthesis of fully-modified 5'-d(TCC-CGC-CTG-TGA) -2'-O-(MOE) - (CAT-GCA-TT) -3' phosphorothioate 20-mer**

- The synthesis of the above sequence is performed on a Millipore Expedite Synthesizer on a 1 micromole scale using
- 30 the 2-cyanoethyl phosphoramidites and CPG support. Detritylation is performed using 3% dichloroacetic acid in methylene chloride. Activation of phosphoramidites is done with a 0.22 M solution of imidazolium or benzimidazolium

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hexafluorophosphate and 0.11 M solution of 1-methylimidazole. Sulfurization is performed using Beaucage reagent. At the end of synthesis, the support is washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

5 Example 67

Synthesis of fully-modified 5'-d(GCC CAA GCT GGC)-2'-O-(MOE)-(ATC CCG TCA)-3' phosphorothioate 20-mer

The synthesis of the above sequence is performed on an Expedite (Millipore) Synthesizer on a 1 micromole scale using
10 the 2-cyanoethyl phosphoramidites and CPG support. Detritylation is performed using 3% dichloroacetic acid in methylene chloride. Activation of phosphoramidites is done with a 0.22 M solution of imidazolium or benzimidazolium hexafluorophosphate and 0.11 M solution of 1-methylimidazole.
15 Sulfurization is performed using Beaucage reagent. At the end of synthesis, the support is washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

Example 68

Synthesis of fully-modified 5'-d(GC^{me}C^{me}-C^{me}AA-GC^{me}T-GGC^{me})-2'-O-
20 (MOE)-(AU^{me}C^{me}-C^{me}GU^{me}-C^{me}A)-3' phosphorothioate 20-mer

The synthesis of the above sequence is performed on an Expedite (Millipore) Synthesizer on a 1 micromole scale using the 2-cyanoethyl phosphoramidites and CPG support. Detritylation is performed using 3% dichloroacetic acid in
25 methylene chloride. Activation of phosphoramidites is done with a 0.22 M solution of imidazolium or benzimidazolium hexafluorophosphate and 0.11 M solution of 1-methylimidazole. Beaucage reagent is used for phosphorothioate synthesis. At the end of synthesis, the support is washed with
30 acetonitrile, cleaved, deprotected and purified in the usual manner.

Example 69**Synthesis of 2'-MOE gapmers**

A 0.1 M solution of 2'-O-MOE amidites are prepared in anhydrous acetonitrile and loaded onto an Expedite Nucleic Acid synthesis system (Millipore) to synthesize oligonucleotides. All other deoxyamidites (A, T, C and G, PerSeptive Biosystem) used in synthesis are also made as 0.1 M solution in anhydrous acetonitrile. All syntheses are carried out in DMT on mode. For the coupling of the 2'-O-MOE amidites coupling time is extended to 10 minutes and this step is carried out twice. All other steps in the protocol supplied by Millipore are used except the extended coupling time (240 seconds). Activation of phosphoramidites is done with a 0.22 M solution of imidazolium or benzimidazolium hexafluorophosphate and 0.11 M solution of 1-methylimidazole. Beaucage reagent is used for phosphorothioate synthesis. The overall coupling efficiencies are expected to be more than 95%. The oligonucleotides are cleaved from the controlled pore glass (CPG) supports and deprotected under standard conditions using concentrated aqueous NH_4OH (30%) at 55°C. 5'-O-DMT containing oligomers are then purified by reverse phase liquid chromatography (C-4, Waters, 7-8 x 300 mm, A=50 mM triethylammonium acetate pH 1, B=100% CH_3CN , 5 to 60% B in 60 minutes). Detritylation with aqueous 80% acetic acid (1 mL, 30 min., room temperature), concentration, followed by desalting by using Sephadese G-25 column will give the oligonucleotides as pure foams. All oligomers are then analyzed by CGE, HPLC and mass spectrometry.

MOE GAPMERS

Sequence 5'-3'	Backbone	Target
T*sT*sC*sT*sC*s GsCsCsCsGsCsTsCs C*sT*sC*sC*sT*sC*sC*	P=S	c-raf
T*sT*sC*sT*sC*s GsCsTsGsGsTsGs	P=S	pkc-a

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AsGs T*sT*sT*sC*sA*		
T*oT*oC*oT*oC*s GsCsCsCsGsCsTsCs C*oT*oC*oC*oT*oC*oC*	P=O, P=S, P=O	c-raf
5 T*oT*oC*oT*oC*s GsCsTsGsGsTsGs AsGs T*oT*oT*oC*oA*	P=O, P=S,	pkc-a

*=2'-O-MOE; All C=5-methyl C;

Example 70

General procedure for uniformly modified 2'-modified

10 oligonucleotide synthesis

2-O-MOE amidites of A, ^{5me}C, G and T are dissolved in anhydrous acetonitrile to give 0.1 M solution. These solutions are loaded onto an Expedite Nucleic Acid Synthesis system (Millipore) to synthesize the oligonucleotides.

15 Activation of phosphoramidites is done with a 0.22 M solution of imidazolium or benzimidazolium hexafluorophosphate and 0.11 M solution of 1-methylimidazole. The coupling efficiencies are expected to be more than 90%. For the coupling of the first amidite coupling time is extended to 10
20 minutes and this step is carried out twice. All other steps in the protocol supplied by Millipore are used except the extended coupling time. Beaucage reagent (0.1 M in acetonitrile) is used as a sulfurizing agent. For diester synthesis, t-BuOOH is used as the oxidizing agent.

25 The oligomers are cleaved from the controlled pore glass (CPG) supports and deprotected under standard conditions using concentrated aqueous NH₄OH (30%) at 55 °C. 5'-O-DMT containing oligomers are then purified by reverse phase high performance liquid chromatography (C-4, Waters, 7.8 x 300 mm,
30 A = 50 mM triethylammonium acetate, pH -7, B = acetonitrile, 5-60% of B in 60 min., flow 1.5 mL/minute). Detritylation with aqueous 80% acetic acid and evaporation, followed by desalting in a Sephadex G-25 column will give the oligonucleotides. Oligonucleotides are analyzed by HPLC, CGE

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and Mass spectrometry.

Sequence	Target
5' T*sC*sT*s G*sA*sG*s T*sA*sG*s C*sA*sG*s A*sG*sG*s A*sG*sC*s T*sC* 3'	I C A M , P=S
5' T*C*T*G*A*G*T*A*G*C*A*G*A*G*G*A*G*C*T*C* 3'	I C A M , P=O

T* = 2'-O-MOE T, A* = 2'-O-MOE A, C* = 2'-O-MOE ^{5me}C, G* = 2'-O-MOE G

Example 71**10 Oligonucleotide synthesis without amino group protection**

Fully-modified 5'-d(TCC-CGC-CTG-TGA-CAT-GCA-TT)-3' phosphorothioate 20 mer; fully modified 5'-d(GCC-CAA-GCT-GGC-ATC-CGT-CA)-3' phosphorothioate 20 mer; fully-modified 5'-d(GCG-TTT-GCT-GCT-CTT-CTT-CTT-GCG)-3' phosphorothioate 21 mer; fully-modified 5'-d(GTT-CTC-GCT-GGT-GAG-TTT-CA)-3' phosphorothioate 20 mer; fully-modified 5'-d(TCC-GTC-ATC-GCT-CCT-CAG-GG)-3' phosphorothioate 20 mer; fully-modified 5'-d(TCC-CGC-CTG-TGA) 2'-O-(MOE)-(CAT-GCA-TT)-3' phosphorothioate 20 mer; fully-modified 5'-d(GCC CAA GCT GGC)-2'-O-(MOE)-(ATC CCG TCA)-3' phosphorothioate 20-mer; fully-modified 5'-d(GC^{me}C^{me}-C^{me}AA-GC^{me}T-GGC^{me})-2'-O-(MOE)-(AU^{me}C^{me}-C^{me}GU^{me}-C^{me}A)-3' phosphorothioate 20 mer; and gapmers are synthesized.

2'-MOE GAPMERS

Sequence 5'-3'	Backbone	Target
T*sT*sC*sT*sC*s GsCsCsGsCsTsCs C*sT*sC*sC*sT*sC*sC*	P=S	c-raf
T*sT*sC*sT*sC*s GsCsTsGsGsTsGs AsGs T*sT*sT*sC*sA*	P=S	pkc-a
T*oT*oC*oT*oC*s GsCsCsGsCsTsCs C*oT*oC*oC*oT*oC*oC*	P=O, P=S, P=O	c-raf
T*oT*oC*oT*oC*s GsCsTsGsGsTsGs AsGs T*oT*oT*oC*oA*	P=O, P=S,	pkc-a

35 * = 2'-O-MOE; All C = 5-methyl C;

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Uniformly 2'-modified oligomers

Sequence	Target
5' T*sC*sT*s G*sA*sG*s T*sA*sG*s C*sA*sG*s A*sG*sG*s A*sG*sC*s T*sC* 3'	I C A M , P=S
5' T*C*T*G*A*G*T*A*G*C*A*G*A*G*G*A*G*C*T*C* 3'	I C A M , P=O

T* = 2'-O-MOE T, A* = 2'-O-MOE A, C* = 2'-O-MOE ^{5me}C, G* = 2'-O-MOE G

Nucleobase unprotected 2'-O-MOE amidites of A, ^{5me}C, G and T and nucleobase unprotected 2'-deoxy amidites of A, C, G and T are dissolved in anhydrous acetonitrile to give 0.1 M solutions. These solutions are loaded onto an Expedite Nucleic Acid Synthesis system (Millipore) to synthesize the oligonucleotides. Activation of phosphoramidites is done with a 0.22 M solution of one of the following activators: pyridinium tetrafluoroborate, pyridinium hexafluoroborate, imidazolium tetrafluoroborate, benzimidazolium tetrafluoroborate, imidazolium hexafluorophosphate or benzimidazolium hexafluorophosphate. After the coupling, any nucleobase N-phosphitylated side product is reverted back by treatment with excess of benzimidazolium triflate in methanol at 25°C for 2 minutes before proceeding to oxidation. The coupling efficiencies are expected to be more than 90%. For the coupling of the first amidite coupling time is extended to 10 minutes and this step is carried out twice. All other steps in the protocol supplied by Millipore are used except the extended coupling time. Beaucage reagent (0.1 M in acetonitrile) is used as a sulfurizing agent. For diester synthesis, t-BuOOH is used as the oxidizing agent.

30 EXAMPLES 72-80

Oligonucleotides with bioreversible protecting groups present in phosphate: Building blocks and oligonucleotide synthesis

Example 72

General Procedures

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All reagents and solvents are purchased from Aldrich Chemical CO. Flash chromatography is performed on silica gel (Baker 40um). Thin layer chromatography is performed on Kieselgel 60 F-254 glass plates from E. Merck and compounds
5 are visualized with UV light and sulfuric acid-methanol spray followed by charring. Solvent systems used for thin-layer chromatography and flash chromatography are: A; ethyl acetate-hexanes 1:1. B; ethyl acetate-hexanes-TEA 2:3:0.5. ¹H and ³¹P spectra are recorded using a Gemini 200 Varian
10 spectrometer. All reactions are performed under an argon atmosphere and solutions rotary evaporated at 35-45°C in vacuo using a vacuum pump-vacuum controller combination.

Example 73

2'-O-MOE-5'-O-(4,4'-dimethoxytrityl)-5-methyluridine (S-
15 pivaloyl-2-thioethyl) bis[N,N-diisopropylphosphoramidite]

To a stirred and precooled solution of 2'-O-MOE-5'-O-(4,4'-dimethoxytrityl)-5-methyluridine (10g, 16 mmol) and diisopropylethylamine (2.7g, 21 mmol) in dry dichloromethane (200 mL) in an ice bath is added dropwise a solution of N,N-
20 (diisopropylamino)chlorophosphine (5.2g, 19 mmol) in dry dichloromethane. The resulting mixture is stirred at room temperature for 55 minutes. Imidazolium triflate (8.0 mmol) is added and a solution of S-(2-hydroxyethyl)thiopivaloate (Tosquellas, G. et al. *Nucleic Acid Res.* 26, 2069, 1998)
25 (3.4g, 21 mmol) in dry dichloromethane is added dropwise over a period of 15 minutes. The reaction mixture is further stirred for 20 hours at room temperature. At the end of this time, the mixture is diluted with dry CH₂Cl₂ (100 mL) and washed with NaHCO₃ (80 mL) and brine 3 times (100 mL) each,
30 dried over MgSO₄ and evaporated to a foam. Flash chromatography using 1:1 Hexanes:EtOAc containing 0.5% triethylamine will yield the title compound.

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Example 74

2'-O-MOE-5'-O-(4,4'-dimethoxytrityl)-5-methyluridine (S-acetyl-2-thioethyl) bis[N,N-diisopropylphosphoramidite] 2'-O-MOE-5'-O-DMT-5-methyluridine

- 5 A solution of 2'-O-MOE-5'-O-DMT-5-methyluridine (10g, 16mmol) and diisopropylethylamine (2.7g, 21mmol) in dry dichloromethane (200 mL) is cooled in an ice bath and stirred for 15 min. Added dropwise a solution of N,N-(diisopropylamino)chlorophosphine (5.2g, 19 mmol) in dry
- 10 CH₂Cl₂. The resulting mixture is stirred at room temperature for 45 minutes. Added imidazolium triflate (8.0 mmol) and a solution of S-(2-hydroxyethyl)-thioacetate (Tosquellas et al. *Nucleic Acids Res.* 26, 2069, 1998) freshly prepared (2.6g, 21 mmol) in dry CH₂Cl₂ in a periods of 10 minutes. The reaction
- 15 mixture is further stirred for 18 hr at RT. At the end of this time, the mixture is diluted with dry CH₂Cl₂ (100 mL) and washed with NaHCO₃ (60 mL) and brine 3 times (80 mL) each and dried over MgSO₄ and evaporated to a solid light yellow foam. Purified by flash chromatography using 1:1
- 20 Hexanes:EtOAc containing 0.5% triethylamine will yield the desired product.

Example 75

2'-deoxy-5'-O-dimethoxytrityl-adenosine-(S-pivaloyl-2-thioethyl) bis[N,N-diisopropylphosphoramidite]

- 25 To a cooled solution of 2'-deoxy-5'-O-dimethyltrityl-adenosine (7.3mmol) and diisopropylamine (1.22g, 9.5mmol) in dry dichloromethane (100ml) stirred in an ice bath, is added a solution of N,N-(diisopropylamino)chlorophosphine (2.33g, 8.76mmol) dropwise in dry CH₂Cl₂. The resulting mixture is
- 30 stirred at RT for 45 min. A solution of S-(2-hydroxyethyl) thiopivaloate (1.42g, 8.76mmol) and imidazolium triflate (3.65mmol) in dry CH₂Cl₂ is added in a periods of 10 min. The reaction mixture is stirred for 22hr at RT. The mixture

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is diluted with CH₂Cl₂ (50 mL) and washed with NaHCO₃ (15 mL) and brine (25 mL) dried over MgSO₄, filtered and evaporated the solvent to a light yellow foam. Purification is done by flash chromatography using Hexanes:EtOAc 1:3 containing 0.5% triethylamine, will yield the desired product.

Example 76

2'-deoxy-5'-O-dimethyltrityl-cytidine-(S-pivaloyl-2-thioethyl) bis[N,N-diisopropylphosphoramidite]

The title compound is prepared as per the procedure described in example 75.

Example 77

2'-deoxy-5'-O-dimethyltrityl-cytidine-(S-benzoyl-2-thioethyl) bis[N,N-diisopropylphosphoramidite]

The title compound is prepared as per the procedure described in example 75.

Example 78

2'-deoxy-5'-O-dimethyltrityl-guanosine-(s-pivaloyl-2-thioethyl) bis[N,N-diisopropylphosphoramidite]

The title compound is prepared as per the procedure described in example 75.

Example 79

2'-deoxy-5'-O-dimethoxytrityl-adenosine-(S-acetyl-2-thioethyl) bis[N,N-diisopropylphosphoramidite]

The title compound is prepared as per the procedure described in example 75.

Example 80

2'-deoxy-5'-O-dimethoxytrityl-cytidine-(S-acetyl-2-thioethyl) bis[N,N-diisopropylphosphoramidite]

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The title compound is prepared as per the procedure described in example 75.

EXAMPLES 81-86

- 5 Oligonucleotides synthesis with the aid of (*S*-Pivaloyl 2-Mercaptoethyl) 3'-O-[(5'-O-(4,4'-Dimethoxytrityl)Thymidyl] *N,N*-Diisopropylphosphoramidite

Example 81

- (*S*-Pivaloyl 2-mercaptoethyl) 3'-O-[(5'-O-(4,4'-dimethoxy-
10 trityl)thymidyl] *N,N*-diisopropylphosphoramidite

- Bis(*N,N*-diisopropylamino)phosphorochloridite (267mg, 1 mmol) in CH₂Cl₂ (2.5 mL) is added to a stirred solution of *S*-pivaloyl 2-mercaptoethanol (162 mg, 1 mmol) and ethyldiisopropylamine (142 mg, 1.1 mmol) in CH₂Cl₂ (1 mL for
15 5 min) at -30°C. The mixture is allowed to warm to room temperature and is stirred for 30 minutes to give *S*-Pivaloyl-2-mercaptoethyl-*N,N,N',N'*-tetraisopropyl phosphorodiamidite. The volume of solution is adjusted to 4.0 mL, an aliquot (320 mL) is taken and added to dry 5'-O-(4,4'-dimethoxytrityl)-
20 thymidine (21.7 mg, 40 mmol). Anhydrous imidazolium triflate (0.45 M in MeCN; 71 mL, 32 mmol) is added, and the mixture is stirred for 40 minutes at room temperature. The reaction is quenched with aqueous NaHCO₃ (5%; 2 mL), diluted with saturated NaCl (5 mL) and extracted with benzene (3x10 mL).
25 The extracts are dried over Na₂SO₄ and evaporated *in vacuo*. The residue is dissolved in 50% aqueous MeCN and purified by reversed phase HPLC on a DeltaPak 15 mm C18 300 column (7.8x300 mm). Isocratic elution with 50% aqueous MeCN for 10 minutes and with 75% aqueous MeCN for 25 minutes at a flow
30 rate 5 mL min⁻¹ is applied. Fractions containing pure are collected, diluted with water (50 mL) and extracted with benzene (5x10 mL). Extracts are dried over Na₂SO₄ and evaporated *in vacuo* to give *S*-pivaloyl 2-mercaptoethyl 3'-O-

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[(5'-O-(4,4'-dimethoxytrityl)thymidyl] *N,N*-diisopropylphosphoramidite.

Example 82

Oligonucleotide synthesis

5 2-(pivaloylthio)ethyl-undecathymidylates are assembled on an ABI 380B DNA Synthesizer using 2-cyanoethyl 3-(4,4'-dimethoxytrityloxy)-3-(2-nitrophenyl)ethyl phosphate, phosphoramidite chemistry, benzimidazolium triflate or imidazolium triflate as the activator, and 3*H*-1,2-benzodithiol-3-one 1,1-dioxide Beaucage reagent (0.1 M in MeCN) as a sulfur-transfer
10 reagent. 5'-O-(4,4'-dimethoxytrityl)thymidyl 2-(pivaloylthio)ethyl *N,N*-diisopropylaminophosphite is employed as a building block. After the synthesis, the oligonucleotide is cleaved from the support photolytically (Guzaev et al.
15 *Bioorg. Med. Chem. Lett.* 8, 1123, 1998).

Deprotection and isolation of oligonucleotides

 The 5'-DMTr protected oligonucleotide is isolated by HPLC (DeltaPak 15 μ C18 300 Å, 3.9x300 mm; 0.1 M NH₄OAc as buffer A, 0.05 M NH₄OAc in 75% aqueous MeCN as buffer B; a
20 linear gradient from 15 to 80 % B in 30 minutes at a flow rate 5.0 mL min⁻¹). The collected fractions are evaporated, treated with 80% aqueous AcOH for 20 minutes, and evaporated to dryness. The residue is desalted on the same column
 eluting first with 0.1 M NaOAc (10 minutes), then with water
25 (10 minutes) and finally eluting as a sodium salt with 50% aqueous MeCN (20 minutes) at a flow rate 5.0 mL min⁻¹.

Example 83

Dodeca[(2-pivaloylthio)ethyl 2'-O-(MOE)-5-methyluridyl phosphate]

30 The title compound is prepared on an ABI 380B synthesizer by using 0.1 M (2-pivaloylthio)ethyl 5'-O-(4,4'-

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dimethoxytrityl)-2'-O-(MOE)-5-methyluridyl *N,N*-diisopropylaminophosphite in MeCN, photolabile solid support from the previous example, 0.45 M imidazolium triflate as an activator, 0.5 M *t*-BuOOH in MeCN as an oxidizer, and 6 minutes coupling time. Upon completeness of the chain assembly (DMTr-Off synthesis) the solid support is dried on an oil pump, placed in a Pyrex test tube and suspended in 80% aqueous MeCN (3 mL). The suspension is degassed, placed in photochemical reactor, and irradiated for 30 minutes at room temperature. The tube is centrifuged, and supernatant is collected. A fresh portion of 80% aqueous MeCN is added. This procedure is repeated for 5 times until less than 4 OD of oligonucleotide material is released after irradiation for 30 minutes. The collected supernatants are diluted with water to get a solution in 30% aqueous MeCN, applied on an HPLC column (DeltaPak 15 μ C18 300 A, 3.9 x 300 mm), and chromatographed in a linear gradient from 25 to 80% MeCN in water for 40 minutes. The main peak is collected and evaporated in vacuo to afford the title compound. An aliquot (5 OD) of the obtained material is treated with concentrated aqueous ammonia (2 mL) for 8 hours at room temperature, evaporated to dryness, and re-dissolved in water (200 μ L). Analysis by capillary electrophoresis (CE) will reveal comigration with authentic sample of dodeca[2'-O-(MOE)-5-methyluridyl phosphate].

Example 84

Dodeca[(2-pivaloylthio)ethyl 2'-O-(MOE)-5-methyluridyl thiophosphate]

The title compound is prepared as described above except that 3*H*-1,2-benzodithiol-3-one 1,1-dioxide (0.05 M in MeCN) is used on an oxidation step as a sulfur transfer reagent. Chromatography on the same column in a linear gradient from 70 to 100% MeCN in water will afford the title compound. After treatment with concentrated aqueous ammonia as above,

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analysis by capillary electrophoresis (CE) will reveal comigration with authentic sample of dodeca[2'-O-(MOE)-5-methyluridyl thiophosphate].

Example 85

5 3'-O-Diglycolyl-5'-(4,4'-dimethoxytrityl)thymidine derivatized CPG

The solid support is prepared according to references Pon, R.T. and Yu, S., *Nucleic Acid Res.* 1997, 25, 3629-3635, and Mullah, B. and Andrus, A., *Tetrahedron Lett.*, 1997, 38, 5751-5754. 5'-(4,4'-Dimethoxytrityl)thymidine (1090 mg, 2.0 mmol), diglycolic anhydride (689 mg, 6.0 mmol), pyridine (10 mL) is stirred for 7 hours at room temperature. The mixture is quenched with water (2 mL) for 10 minutes and evaporated to an oil. The residue is dissolved in ethyl acetate (50 mL), washed with triethylammonium acetate (2 M aqueous, 5 x 10 mL), then with water (5 x 10 mL), dried over Na₂SO₄ and evaporated. The residue is dissolved in pyridine (10 mL), long chain alkyl amine Controlled Pore Glass (CPG, 3.0g) is added and the mixture is degassed *in vacuo*. *N,N'*-diisopropylcarbodiimide (800 mg, 6.3 mmol) is added, and the mixture is shaken overnight at room temperature. The solid support is filtered out, treated with a mixture of acetic anhydride, *N*-methylimidazole, 2,6-lutidine and THF (1:1:2:16 v/v) for 30 minutes, filtered, washed on filter with acetonitrile (5 x 10 mL) and dried on an oil pump. Efficiency of the derivatization is determined by dimethoxytrityl assay to show the loading which is expected to be about 60 $\mu\text{mol g}^{-1}$.

Example 86

30 Oligonucleotide synthesis

Chimerical oligothymidylates are assembled on an ABI 380B DNA Synthesizer using 5'-O-(4,4'-

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dimethoxytrityl)thymidine 3'-O-(carboxymethyloxy)acetate derivatized CPG (diglycolyl-T CPG)¹ (Scheme 1), phosphoramidite chemistry, and either commercial oxidizer for 3H-1,2-benzodithiol-3-one 1,1-dioxide (0.05 M in MeCN) as the sulfur-transfer reagent. Either 5'-O-(4,4'-dimethoxytrityl)thymidyl 2-(pivaloylthio)ethyl N,N'-diisopropylaminophosphite or 3'-O-[5-methyl-2-O-(MOE)-5'-O-(4,4'-dimethoxytrityl)uridyl] 2-(pivaloylthio)ethyl N,N'-diisopropylaminophosphite are employed for chain assembly to create 2-(pivaloylthio)ethyl triester internucleosidic moieties. After extensive washing with MeCN and drying the oligonucleotide is released from the solid support by treatment with 0.01 M K₂CO₃ in MeOH (2x5 mL and 2x20 mL for 1 and 15 μ mol syntheses, respectively). Each portion is passed back and forth through the column for 45 minutes, neutralized by passing through short column with Dowex 50Wx8 (PyH⁺; ca. 1 mL). The combined eluates are evaporated to dryness, co-evaporated with MeCN (10 mL), and dissolved in water. The obtained mixture consists of along with products of methanolysis of 2-(pivaloylthio)ethyl groups (ca. 1 to 1.5% of methanolysis per each group). Target oligonucleotide is isolated by RP HPLC on Delta Pak 15 μ m C18 300Å column (3.9 x 300 mm and 7.8 x 300 mm for 1 and 15 μ mol syntheses, respectively), using 0.1 M NH₄OAc as buffer A, 80% aq MeCN as buffer B, and a linear gradient from 0 to 100% B in 50 minutes at a flow rate 1.5 and 5 mL min⁻¹, respectively. Collected fractions are evaporated, redissolved in water and desalted by injecting onto the same column, then washing with water (10 minutes) and eluting an oligonucleotide as an ammonium salt with 50% aq MeCN (20 minutes). Homogeneity of oligonucleotides is characterized by RP HPLC, mass spectrometry and ³¹P NMR.

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Example 87**Synthesis of Bioreversible (SATE) Oligonucleotides without exocyclic amine protection using the activators**

Synthetic oligonucleotides as shown in Example 71, with
5 (S-pivaloyl 2-mercaptoethyl)bioreversible phosphate
protecting groups for the internucleotide phosphate linkages,
are synthesized with the aid of (S-pivaloyl 2-mercaptoethyl)
3'-O-[(5'-O-(4,4'-dimethoxytrityl)thymidyl] N,N-diisopropyl-
phosphoramidite, (S-pivaloyl 2-mercaptoethyl) 3'-O-[(5'-O-
10 (4,4'-dimethoxytrityl)adenyl] N,N-Diisopropylphosphoramidite,
(S-pivaloyl 2-mercaptoethyl) 3'-O-[(5'-O-(4,4'-
dimethoxytrityl)cytidyl] N,N-diisopropylphosphoramidite, and
(S-pivaloyl 2-mercaptoethyl) 3'-O-[(5'-O-(4,4'-
dimethoxytrityl)guanyl] N,N-diisopropylphosphoramidite
15 without exocyclic amine protection. Oligonucleotides are
assembled on an ABI 380B DNA Synthesizer using 5'-O-(4,4'-
dimethoxytrityl)nucleoside 3'-O-(carboxymethyloxy)acetate
derivatized CPG (diglycolyl-Nucleoside- CPG), phosphoramidite
chemistry, 3H-1,2-benzodithiol-3-one 1,1-dioxide (0.05 M in
20 MeCN) as the sulfur-transfer reagent, t-BuOOH as the
oxidizing agent for phosphodiester linkages. One of the
following activators as a 0.22 M solution along with a 0.11 M
solution of N-methyl-imidazole in acetonitrile is used as the
activator: pyridinium tetrafluoroborate, pyridinium
25 hexafluorophosphate, imidazoliumtetrafluoroborate, imidazolim
hexafluorophosphate, benzimidazolium tetrafluoroborate, or
benzimidazolium hexafluorophosphate, imidazolium triflate, or
benzimidazolium triflate. The small amount of nucleobase N-
phosphitylated side product is reverted back to the free
30 nucleoside derivative by treating the reaction solid support
with excess of benzimidazolium triflate in methanol at
ambient temperature for 2-3 minutes. After completeness of
oligonucleotide synthesis, the column is washed with dioxane
(10 mL) to give pivaloyl-containing oligonucleosides still on

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the solid support. After extensive washing with MeCN and drying, the oligonucleotide is released from the solid support by treatment with 0.01 M K_2CO_3 in MeOH (2x5 mL and 2x20 mL for 1 and 15 μ mol syntheses, respectively). Each
5 portion is passed back and forth through the column for 45 minutes, neutralized by passing through short column with Dowex 50Wx8 (PyH⁺; ca. 1 mL). The combined eluates are evaporated to dryness, co-evaporated with MeCN (10 mL), and dissolved in water. The obtained mixture includes the
10 products of methanolysis of 2-(pivaloylthio)ethyl groups (ca. 1 to 1.5% of methanolysis per each group). Target oligonucleotide is isolated by RP HPLC on Delta Pak 15 μ m C18 300Å column (3.9 x 300 mm and 7.8 x 300 mm for 1 and 15 μ mol syntheses, respectively), using 0.1 M NH_4OAc as buffer A, 80%
15 aq MeCN as buffer B, and a linear gradient from 0 to 100% B in 50 minutes at a flow rate 1.5 and 5 mL min⁻¹, respectively. Collected fractions are evaporated, redissolved in water and desalted by injection onto the same column, then washing with water (10 minutes) and finally
20 elution as the ammonium salt with 50% aq MeCN (20 minutes). Homogeneity of chimerical oligonucleotides is characterized by RP HPLC and capillary electrophoresis, and their structure is confirmed by mass spectrometry and ³¹P NMR.

It is intended that each of the patents, applications,
25 printed publications, and other published documents mentioned or referred to in this specification be herein incorporated by reference in their entirety.

Those skilled in the art will appreciate that numerous changes and modifications may be made to the preferred
30 embodiments of the invention and that such changes and modifications may be made without departing from the spirit of the invention. It is therefore intended that the appended claims cover all such equivalent variations as fall within the true spirit and scope of the invention.

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What is claimed is:

1. A method for preparing a phosphitylated compound comprising the steps of:

providing a compound having a hydroxyl group; and

5 reacting said compound with a phosphitylating reagent in the presence of a pyridinium salt in a solvent under conditions of time, temperature and pressure effective to yield said phosphitylated compound.

2. The method of claim 1 wherein said compound is a
10 nucleoside.

3. The method of claim 2 wherein said compound is a 5'-O- protected nucleoside having a 3' hydroxyl group.

4. The method of claim 1 wherein said compound is a nucleoside dimer having a 3' or 5' hydroxyl group.

15 5. The method of claim 1 wherein said compound is an oligonucleotide or oligonucleotide analog having a 3' or 5' hydroxyl group.

6. The method of claim 2 wherein said nucleoside has a 5' or a 2' hydroxyl group.

20 7. The method of claim 1 wherein said phosphitylating reagent is 2-cyanoethyl-N,N,N',N'-tetraisopropylphosphorodiamidite, bis(N,N-diisopropylamino)-2-methyl-trifluoroacetylaminooethoxyphosphine or bis(N,N-diisopropylamino)-2-diphenylmethylsilylethoxyphosphine.

25

8. The method of claim 1 wherein said pyridinium salt is pyridinium hydrochloride, pyridinium trifluoroacetate or pyridinium dichloroacetate.

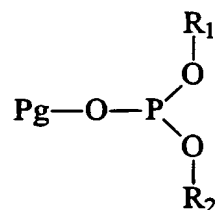
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9. The method of claim 1 wherein said solvent is dichloromethane, acetonitrile, ethyl acetate, tetrahydrofuran or a mixture thereof.

5 10. The method of claim 1, wherein said activator is bound to a solid support.

11. The method of claim 10 wherein said activator is a polyvinyl pyridinium salt.

10 12. A method for the preparation of a compound of formula:



wherein:

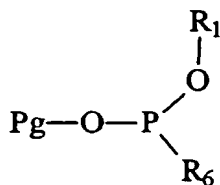
R_1 is a nucleoside or an oligonucleotide;

15 R_2 is a nucleoside linked to a solid support, or an oligonucleotide linked to a solid support;

Pg is a phosphorus protecting group;

comprising:

providing a phosphoramidite of formula:



wherein

R_6 is $-\text{N}(\text{R}_7)_2$ wherein R_7 is alkyl having from one to about six carbons; or R_7 is a heterocycloalkyl or heterocycloalkenyl ring containing from 4 to 7 atoms, and

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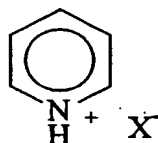
having up to 3 heteroatoms selected from nitrogen, sulfur, and oxygen; and

reacting said phosphoramidite with a hydroxyl group of a nucleoside linked to a solid support, or an oligonucleotide
5 linked to a solid support;

said reaction being performed in the presence of an activating reagent, said activating reagent comprising at least one pyridinium salt and at least one substituted imidazole.

10

13. The method of claim 12 wherein said pyridinium salt has the formula



where X^- is trifluoroacetate, ^-O -mesyl, ^-O -tosyl, ^-Br ,
15 ^-O -trifluorosulfonyl, hexafluorophosphate or tetrafluoroborate.

14. The method of claim 13 wherein the substituted imidazole is 1-methylimidazole.

15. The method of claim 13 wherein X^- is
20 trifluoroacetate.

16. The method of claim 12 wherein the phosphoramidite is reacted with the 5'-hydroxyl of a solid-support bound nucleoside or oligonucleotide.

17. The method of claim 12 wherein the oligonucleotide
25 comprises phosphorothioate intersugar linkages.

18. A method for the preparation of an oligonucleotide

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comprising the steps of:

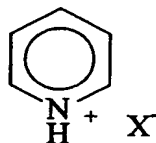
providing a 3'-nucleoside phosphoramidite or 3'-oligonucleotide phosphoramidite; and

5 reacting said 3'-nucleoside phosphoramidite or 3'-oligonucleotide phosphoramidite with the 5'-hydroxyl of a nucleoside, or oligonucleotide in the presence of an activating reagent;

said nucleoside or oligonucleotide being optionally bound to a solid support;

10 said activating reagent comprising at least one pyridinium salt and one substituted imidazole.

19. The method of claim 18 wherein the pyridinium salt has the formula



15 where X⁻ is trifluoroacetate, ⁻O-mesyl, ⁻O-tosyl, ⁻Br, ⁻O-trifluorosulfonyl, hexafluorophosphate or tetrafluoroborate.

20. The method of claim 19 wherein the substituted imidazole is 1-methylimidazole.

20 21. The method of claim 20 wherein X⁻ is trifluoroacetate.

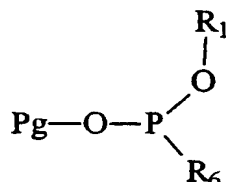
22. The method of claim 20 wherein the 3'-mononucleoside phosphoramidite or 3'-oligonucleotide phosphoramidite is reacted with the 5'-hydroxyl of a solid-
25 support bound nucleoside, nucleotide or oligonucleotide.

23. The method of claim 20 wherein the oligonucleotide

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comprises phosphorothioate intersugar linkages.

24. A synthetic method comprising:
providing a phosphoramidite of formula:



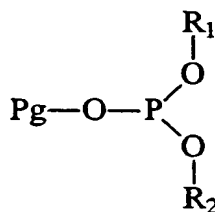
5 wherein:

R_1 is a nucleoside or an oligonucleotide;

R_6 is $-\text{N}(\text{R}_7)_2$ wherein R_7 is alkyl having from one to about six carbons; or R_7 is a heterocycloalkyl or heterocycloalkenyl ring containing from 4 to 7 atoms, and
10 having up to 3 heteroatoms selected from nitrogen, sulfur, and oxygen;

Pg is a phosphorus protecting group;

and reacting said phosphoramidite with a hydroxyl group of a nucleoside linked to a solid support, or an
15 oligonucleotide linked to a solid support, to form a compound of formula:



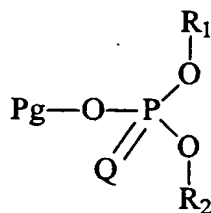
wherein:

R_2 is a nucleoside linked to a solid support, or an
20 oligonucleotide linked to a solid support;

said reaction being performed in the presence of an activating reagent, said activating reagent comprising at least one pyridinium salt and one substituted imidazole; and oxidizing or sulfurizing said compound to form a

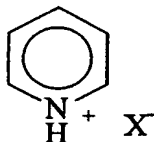
- 127 -

compound of formula:



wherein Q is O or S.

25. The method of claim 24 wherein the pyridinium salt
5 has the formula



where X⁻ is trifluoroacetate, ⁻O-mesyl, ⁻O-tosyl, ⁻Br,
⁻O-trifluorosulfonyl, hexafluorophosphate or
tetrafluoroborate.

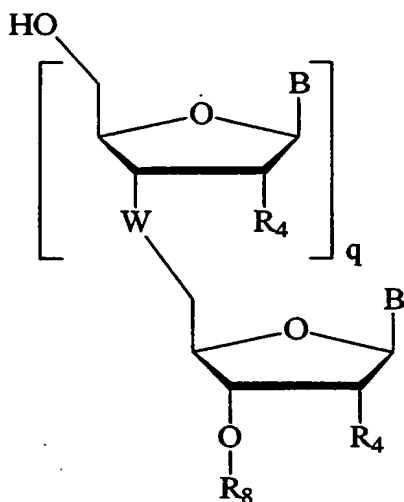
10 26. The method of claim 25 wherein the substituted
imidazole is 1-methylimidazole.

27. The method of claim 26 wherein X⁻ is
trifluoroacetate.

28. The method of claim 16 wherein Q is S.

15 29. A synthetic method comprising:
providing a compound of Formula X:

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X

wherein:

B is a nucleobase;

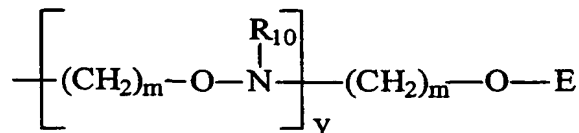
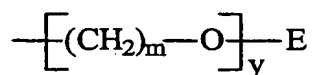
5 R_8 is H, a hydroxyl protecting group, or a linker connected to a solid support;

W is an optionally protected internucleoside linkage;

q is 0 to about 50;

 R_4 is H, F, O-R, S-R or N-R(R_{10});

10 R is H, a protecting group, or has one of the formulas:



where

each m is independently from 1 to 10;

15 y is from 0 to 10;

E is H, a hydroxyl protecting group, C_1 - C_{10} alkyl, N(R_{10}) (R_{11}) or N=C(R_{10}) (R_{11}); substituted or unsubstituted C_1 - C_{10} alkyl, C_2 - C_{10} alkenyl, C_2 - C_{10} alkynyl, wherein the substitutions are selected from one or several halogen,

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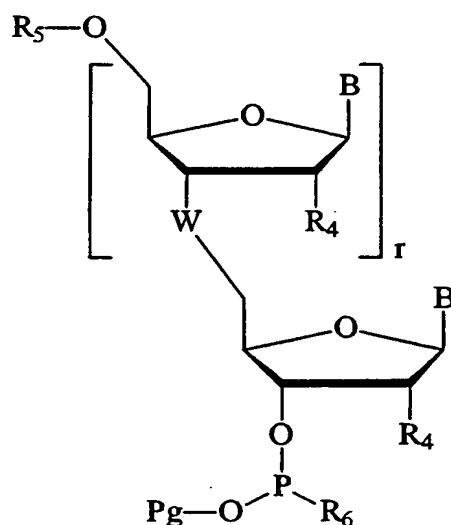
cyano, carboxy, hydroxy, nitro and mercapto residues; and

each R_{10} or R_{11} is, independently, H, substituted or unsubstituted C_1 - C_{10} alkyl, C_2 - C_{10} alkenyl, C_2 - C_{10} alkynyl, wherein the substitutions are selected from one or several

5 halogen, cyano, carboxy, hydroxy, nitro and mercapto residues; alkylthioalkyl, a nitrogen protecting group, or R_{10} and R_{11} , together, are a nitrogen protecting group or wherein R_{10} and R_{11} are joined in a ring structure that can include at least one heteroatom selected from N and O;

10 or R is $-\text{CH}_2-\text{CH}_2-\text{O}-\text{CH}_2-\text{CH}_2-\text{N}(\text{R}_{10})(\text{R}_{11})$;

reacting the compound of Formula X in the presence of an activating reagent with a compound of Formula XI:



XI

where r is 0 to about 50;

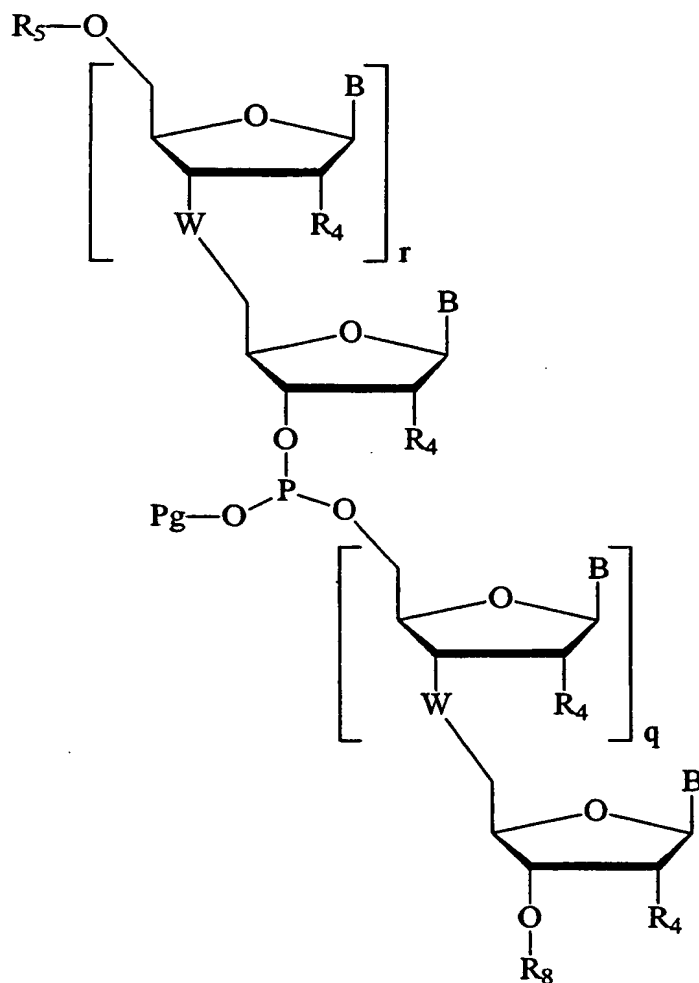
R_5 is a hydroxyl protecting group;

R_6 is $-\text{N}(\text{R}_7)_2$ wherein R_7 is alkyl having from one to about six carbons; or R_7 is a heterocycloalkyl or

20 heterocycloalkenyl ring containing from 4 to 7 atoms, and having up to 3 heteroatoms selected from nitrogen, sulfur, and oxygen;

to form a compound of Formula XII:

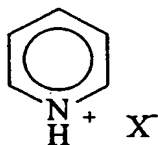
- 130 -



XII

wherein said activating reagent comprises at least one pyridinium salt and one substituted imidazole.

- 5 30. The method of claim 29 wherein the pyridinium salt has the formula:



where X^- is trifluoroacetate, ^-O -mesyl, ^-O -tosyl, ^-Br , or ^-O -trifluorosulfonyl, hexafluorophosphate or
 10 tetrafluoroborate.

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31. The method of claim 30 wherein the substituted imidazole is 1-methylimidazole.

32. The method of claim 31 wherein X^- is trifluoroacetate.

5 33. The method of claim 29 wherein R_8 is a linker connected to a solid support.

34. The method of claim 29 wherein R_4 is $-O-R$ wherein R has the formula $-[-(CH_2)_m-O-]_y-E$; m is 2, y is 1; and E is CH_3 , $-N(R_{10})(R_{11})$, or $-CH_2-CH_2-N(R_{10})(R_{11})$.

10 35. The method of claim 29 wherein r is 0.

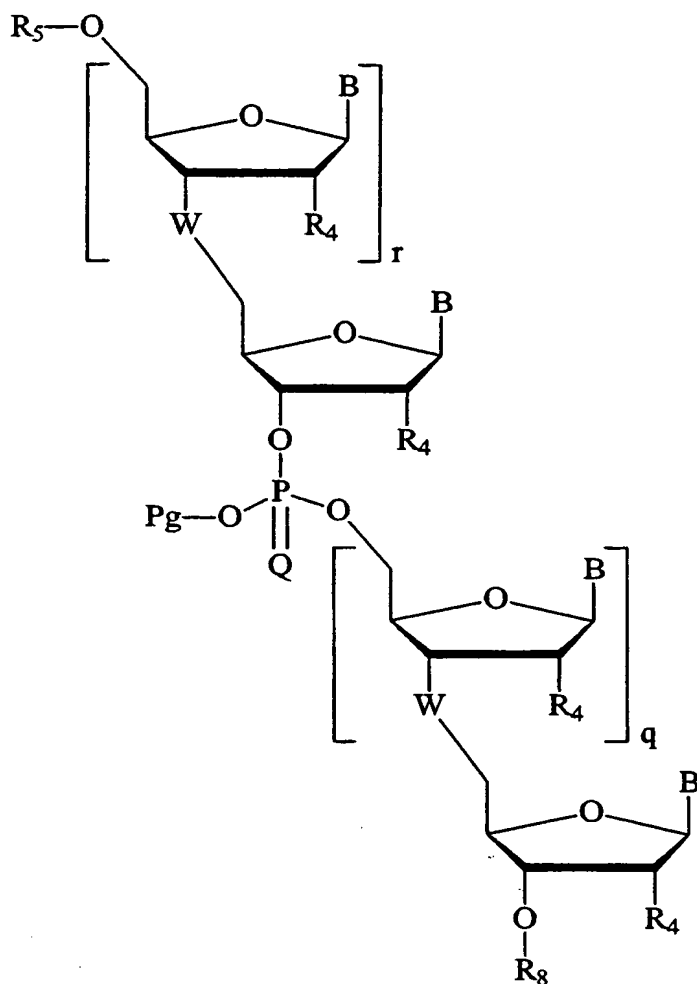
36. The method of claim 29 wherein R_6 is diisopropylamino.

37. The method of claim 29 wherein Pg is $-CH_2CH_2CN$, $-CH_2CH=CHCH_2CN$, para- $CH_2C_6H_4CH_2CN$, $-(CH_2)_{2-5}N(H)COCF_3$,
15 $-CH_2CH_2Si(C_6H_5)_2CH_3$, or $-CH_2CH_2N(CH_3)COCF_3$.

38. The method of claim 29 wherein Pg is $-CH_2CH_2CN$.

39. The method of claim 29 further comprising oxidizing or sulfurizing the compound of Formula XII to form a compound of Formula XIII:

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XIII

where Q is O or S.

40. The method of claim 39 further comprising a capping
5 step.

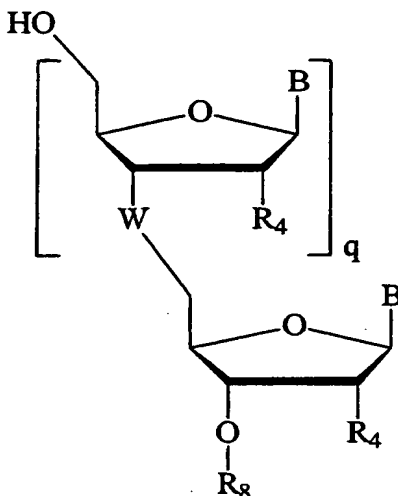
41. The method of claim 40 wherein the capping step is
performed prior to oxidation.

42. The method of claim 41 further comprising the step
of cleaving the oligomeric compound to produce a further
10 compound of formula X.

43. A synthetic method comprising:

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providing a compound of Formula X:



X

5 wherein:

B is a nucleobase;

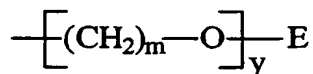
R₈ is H, a hydroxyl protecting group, or a linker connected to a solid support;

W is an optionally protected internucleoside linkage;

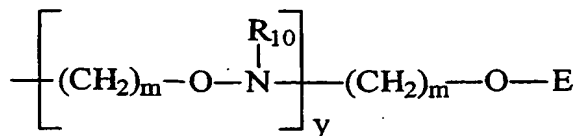
10 q is 0 to about 50;

R₄ is H, F, O-R, S-R or N-R(R₁₀);

R is H, a protecting group, or has one of the formulas:



15



where

each m is independently from 1 to 10;

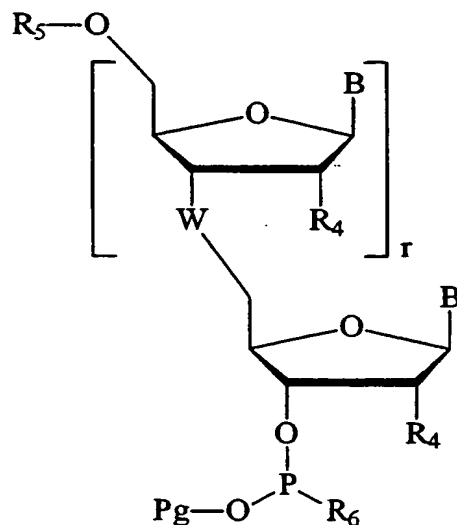
y is from 0 to 10;

E is H, a hydroxyl protecting group, C₁-C₁₀ alkyl,

20 N(R₁₀)(R₁₁) or N=C(R₁₀)(R₁₁); substituted or unsubstituted

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- C_1 - C_{10} alkyl, C_2 - C_{10} alkenyl, C_2 - C_{10} alkynyl, wherein the substitutions are selected from one or several halogen, cyano, carboxy, hydroxy, nitro and mercapto residues; and each R_{10} or R_{11} is, independently, H, substituted or unsubstituted C_1 - C_{10} alkyl, C_2 - C_{10} alkenyl, C_2 - C_{10} alkynyl, wherein the substitutions are selected from one or several halogen, cyano, carboxy, hydroxy, nitro and mercapto residues; alkylthioalkyl, a nitrogen protecting group, or R and R_{10} , together, are a nitrogen protecting group or wherein R and R_2 are joined in a ring structure that can include at least one heteroatom selected from N and O;
- or R is $-\text{CH}_2-\text{CH}_2-\text{O}-\text{CH}_2-\text{CH}_2-\text{N}(\text{R}_{10})(\text{R}_{11})$;
- provided that R_{14} is not H or OH;
- reacting the compound of Formula X in the presence of an activator with a compound of Formula XI:

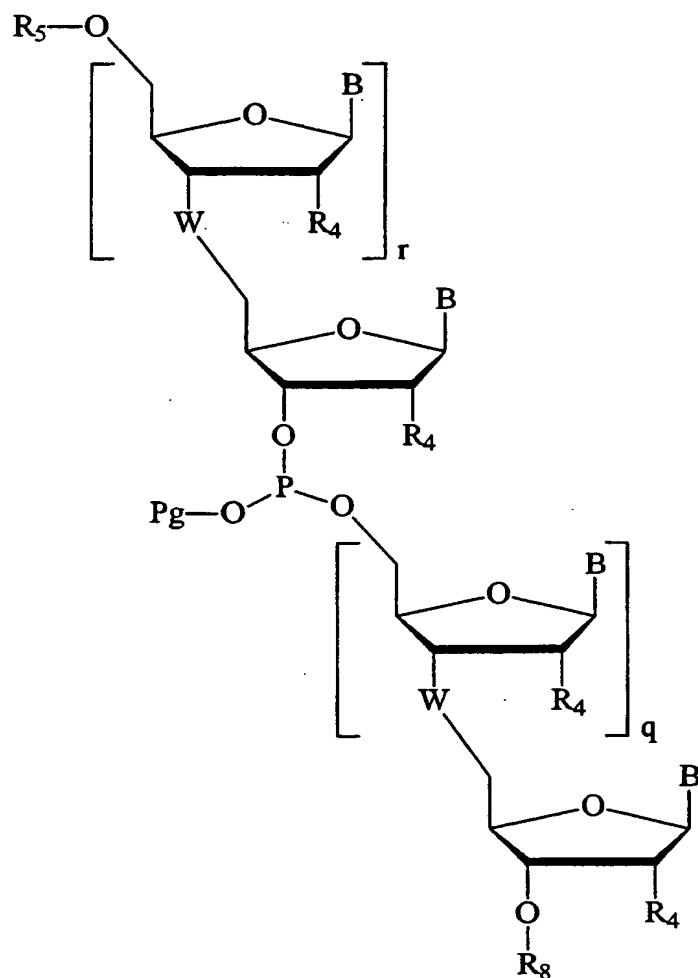


XI

- where r is 0 to about 50;
- R_5 is a hydroxyl protecting group;
- R_6 is $-\text{N}(\text{R}_7)_2$ wherein R_7 is alkyl having from one to about six carbons; or R_7 is a heterocycloalkyl or heterocycloalkenyl ring containing from 4 to 7 atoms, and having up to 3 heteroatoms selected from nitrogen, sulfur,

and oxygen;

to form a compound of Formula XII:



XII

5 wherein the activator has the formula G^+U^- ,

where

G^+ is selected from the group consisting of pyridinium, imidazolium, and benzimidazolium; and

U^- is selected from the group consisting of
 10 hexafluorophosphate, tetrafluoroborate, triflate, hydrochloride, trifluoroacetate, dichloroacetate, ^-O -mesyl, ^-O -tosyl, ^-Br , and ^-O -trifluorosulfonyl.

44. The method of claim 43 wherein R_8 is a linker

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connected to a solid support.

45. The method of claim 44 wherein R_4 is $-O-R$ wherein R has the formula $-[-(CH_2)_m-O-]_y-E$; m is 2, y is 1; and E is CH_3 , $-N(R_{10})(R_{11})$, or $-CH_2-CH_2-N(R_{10})(R_{11})$.

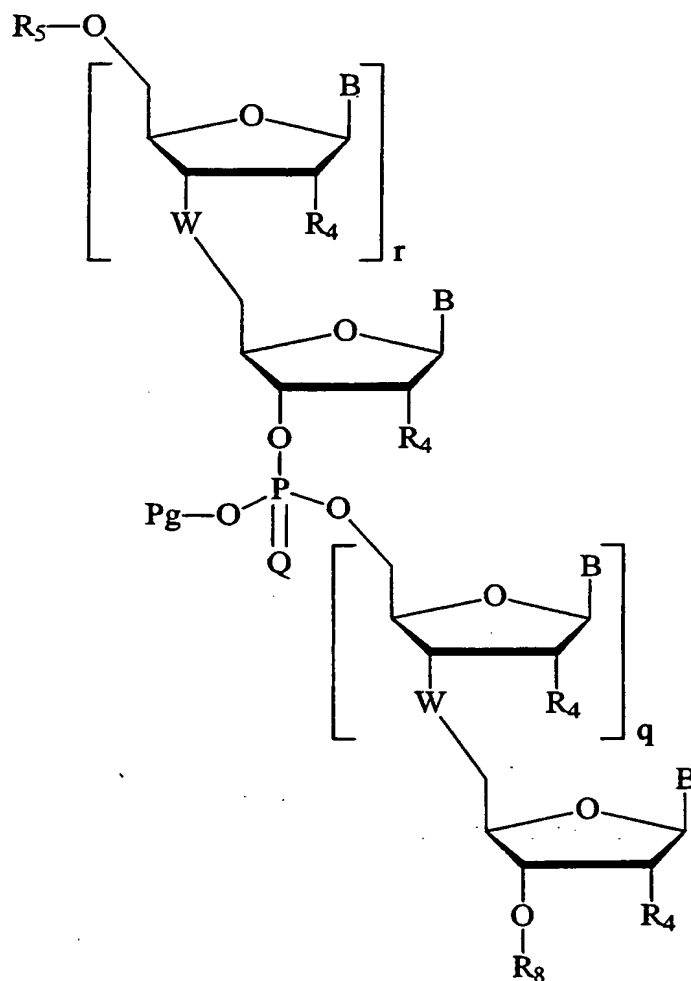
5 46. The method of claim 43 wherein r is 0.

47. The method of claim 43 wherein R_6 is diisopropylamino.

48. The method of claim 43 wherein Pg is $-CH_2CH_2CN$,
 $-CH_2CH=CHCH_2CN$, $para-CH_2C_6H_4CH_2CN$, $-(CH_2)_{2-5}N(H)COCF_3$,
10 $-CH_2CH_2Si(C_6H_5)_2CH_3$, or $-CH_2CH_2N(CH_3)COCF_3$.

49. The method of claim 43 wherein Pg is $-CH_2CH_2CN$.

50. The method of claim 43 further comprising oxidizing or sulfurizing the compound of Formula XII to form a compound of Formula XIII:



XIII

where Q is O or S.

51. The method of claim 50 further comprising a capping step.

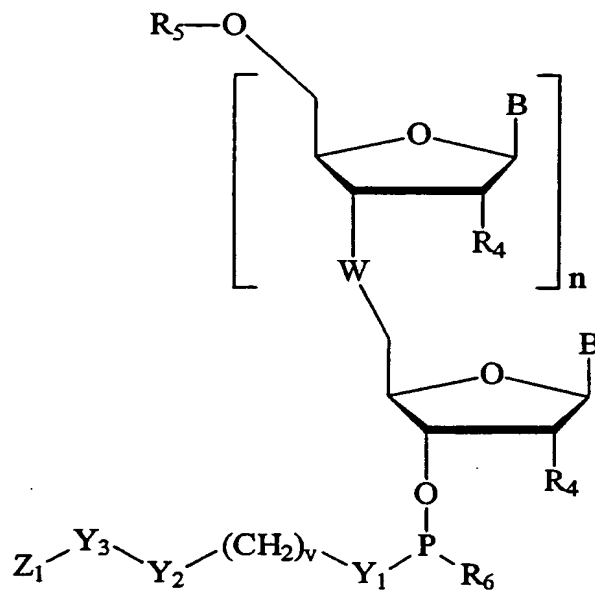
52. The method of claim 51 wherein the capping step is performed prior to oxidation.

53. The method of claim 52 further comprising the step of cleaving the oligomeric compound to produce a further compound of Formula X.

54. A synthetic method comprising:

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providing a compound of Formula XX:

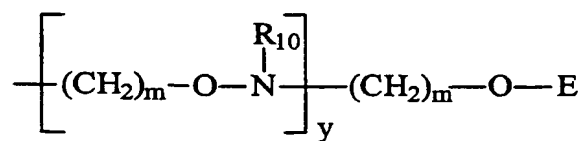
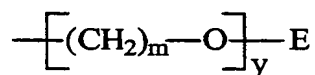


XX

wherein:

5 R_4 is H, F, O-R, S-R or N-R(R_{10});

R is H, a protecting group, or has one of the formulas:



where

10 each m is independently from 1 to 10;

y is from 0 to 10;

E is H, a hydroxyl protecting group, C_1 - C_{10} alkyl,

$N(R_{10})(R_{11})$ or $N=C(R_{10})(R_{11})$; substituted or unsubstituted

C_1 - C_{10} alkyl, C_2 - C_{10} alkenyl, C_2 - C_{10} alkynyl, wherein the

15 substitutions are selected from one or several halogen,

cyano, carboxy, hydroxy, nitro and mercapto residues; and

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each R_{10} or R_{11} is, independently, H, substituted or unsubstituted C_1 - C_{10} alkyl, C_2 - C_{10} alkenyl, C_2 - C_{10} alkynyl, wherein the substitutions are selected from one or several halogen, cyano, carboxy, hydroxy, nitro and mercapto

5 residues; alkylthioalkyl, a nitrogen protecting group, or R_{10} and R_{11} , together, are a nitrogen protecting group or wherein R_{10} and R_{11} are joined in a ring structure that can include at least one heteroatom selected from N and O;

or R is $-\text{CH}_2-\text{CH}_2-\text{O}-\text{CH}_2-\text{CH}_2-\text{N}(\text{R}_{10})(\text{R}_{11})$;

10 R_5 is a hydroxyl protecting group;

Z_1 is aryl having 6 to about 14 carbon atoms or alkyl having from one to about six carbon atoms;

Y_1 is O or S;

Y_2 is O or S;

15 Y_3 is $\text{C}(=\text{O})$ or S;

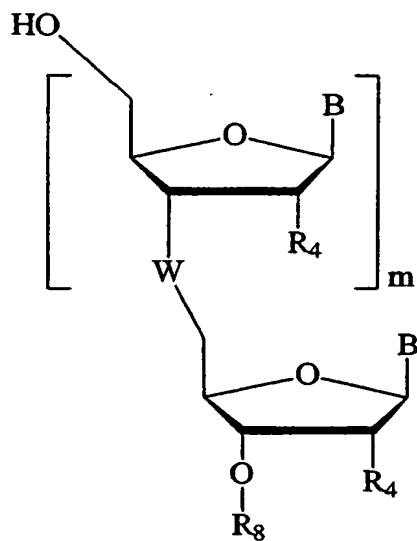
v is 2 to about 4;

B is a nucleobase;

R_6 is $-\text{N}(\text{R}_7)_2$ wherein R_7 is alkyl having from one to about six carbons; or R_7 is a heterocycloalkyl or
20 heterocycloalkenyl ring containing from 4 to 7 atoms, and having up to 3 heteroatoms selected from nitrogen, sulfur, and oxygen;

reacting said compound of Formula XX with a compound of Formula XXI:

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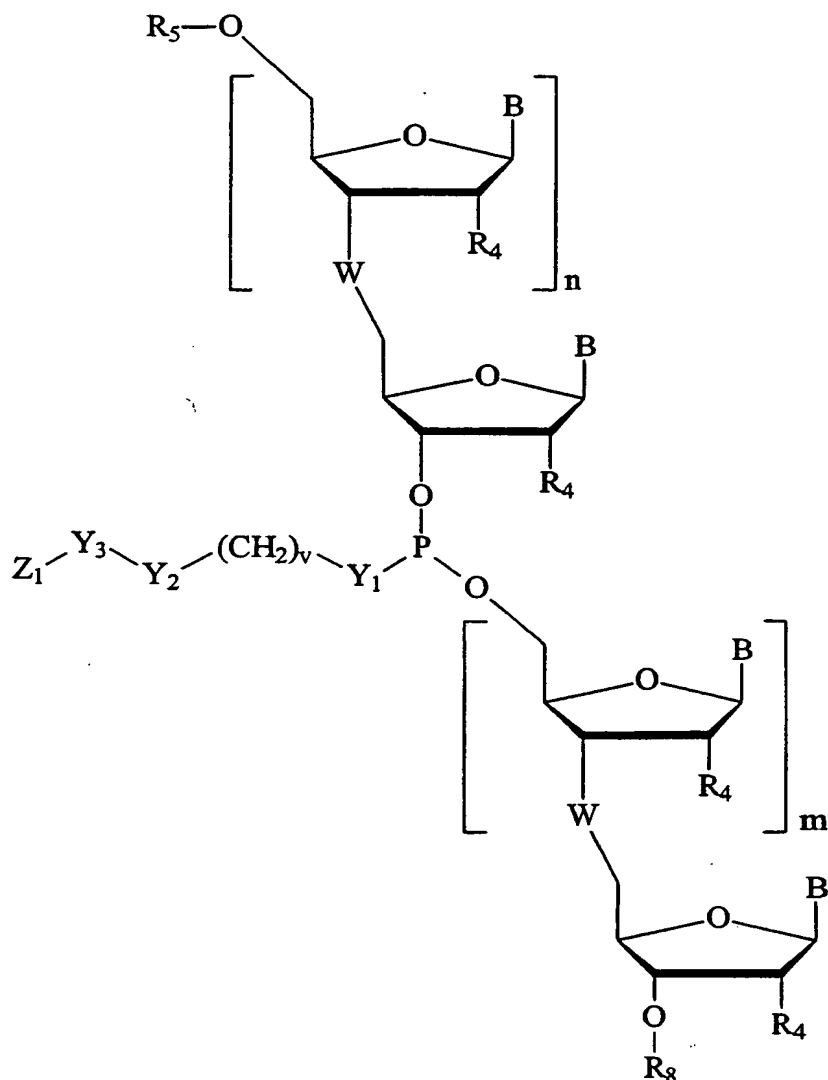
XXI

wherein:

R₈ is H, a hydroxyl protecting group, or a linker
5 connected to a solid support;

in the presence of an activator to form a compound of
Formula XXII:

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XXII

wherein said activator has the formula G^+U^- , where:

G^+ is selected from the group consisting of pyridinium,
 5 imidazolium, and benzimidazolium; and

U^- is selected from the group consisting of
 hexafluorophosphate, tetrafluoroborate, triflate,
 hydrochloride, trifluoroacetate, dichloroacetate, ^-O -mesyl,
 ^-O -tosyl, ^-Br , and ^-O -trifluorosulfonyl;

10 or said activator is a substituted imidazolium triflate.

55. The method of claim 54 wherein said activator is

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imidazolium triflate.

56. The method of claim 55 wherein v is 2; and Y_3 is $C(=O)$.

57. The method of claim 56 wherein Z is methyl, phenyl
5 or t -butyl.

58. The method of claim 57 wherein Z is t -butyl.

59. The method of claim 56 wherein n is 0.

60. The method of claim 56 wherein R_2 is a linker to a
10 solid support.

61. The method of claim 56 wherein Y_1 and Y_2 are each
O.

62. The method of claim 56 wherein Y_1 and Y_2 are each
S.

15 63. The method of claim 56 wherein Y_1 is O and Y_2 is S.

64. The method of claim 56 wherein each R_6 is
isopropyl.

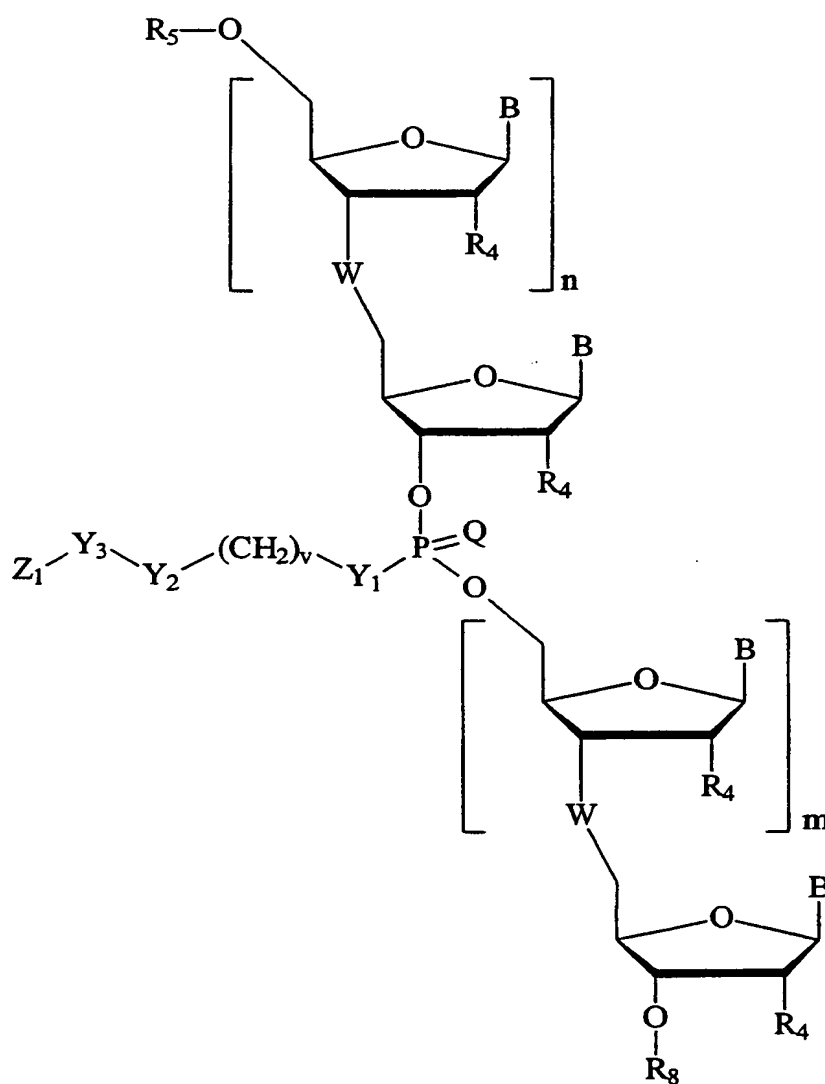
65. The method of claim 56 wherein n is 0; R_3 is H,
 R_5 is diisopropylamino; Y_1 is O; Y_2 is S; and Z is methyl or
20 t -butyl.

66. The method of claim 65 wherein Z is t -butyl.

67. The method of claim 55 wherein each B is devoid of
exocyclic amine protection.

68. The method of claim 55 wherein W is an optionally protected phosphodiester, phosphorothioate, phosphorodithioate, or alkyl phosphonate internucleotide linkage.

5 69. The method of claim 55 further comprising oxidizing or sulfurizing the compounds of Formula XXII to form a compound of Formula XXIII:



10 where Q is O or S.

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70. The method of claim 69 further comprising a capping step.

71. The method of claim 70 wherein the capping step is performed prior to oxidation.

5 72. The method of claim 71 further comprising the step of cleaving the oligomeric compound to produce a further compound of Formula XXI.

73. The method of claim 43 wherein G^+ is pyridinium and U^- is hexafluorophosphate or tetrafluoroborate.

10 74. The method of claim 73 wherein U^- is hexafluorophosphate.

75. The method of claim 43 wherein G^+ is imidazolium or benzimidazolium and U^- is selected from the group consisting of hexafluorophosphate, tetrafluoroborate, triflate,
15 hydrochloride, trifluoroacetate, dichloroacetate, ^-O -mesyl, ^-O -tosyl, ^-Br , and ^-O -trifluorosulfonyl.

76. The method of claim 75 wherein G^+ is imidazolium or benzimidazolium and U^- is selected from the group consisting of hexafluorophosphate, tetrafluoroborate, and triflate.

20 77. The method of claim 75 wherein G^+ is imidazolium or benzimidazolium and U^- is selected from the group consisting of hydrochloride, trifluoroacetate, dichloroacetate, ^-O -mesyl, ^-O -tosyl, ^-Br , and ^-O -trifluorosulfonyl.

78. The method of claim 43 wherein G^+ is imidazolium
25 and U^- is selected from the group consisting of hexafluorophosphate, tetrafluoroborate, triflate, hydrochloride, trifluoroacetate, dichloroacetate, ^-O -mesyl,

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O -tosyl, Br , and O -trifluorosulfonyl.

79. The method of claim 78 wherein U^- is selected from the group consisting of hexafluorophosphate, tetrafluoroborate, and triflate.

5 80. The method of claim 78 wherein U^- is selected from the group consisting of hydrochloride, trifluoroacetate, dichloroacetate, O -mesyl, O -tosyl, Br , and O -trifluorosulfonyl.

10 81. The method of claim 43 wherein G^+ is benzimidazolium and U^- is selected from the group consisting of hexafluorophosphate, tetrafluoroborate, triflate, hydrochloride, trifluoroacetate, dichloroacetate, O -mesyl, O -tosyl, Br , and O -trifluorosulfonyl.

15 82. The method of claim 81 wherein U^- is selected from the group consisting of hexafluorophosphate, tetrafluoroborate, and triflate.

20 83. The method of claim 81 wherein U^- is selected from the group consisting of hydrochloride, trifluoroacetate, dichloroacetate, O -mesyl, O -tosyl, Br , and O -trifluorosulfonyl.

84. The method of claim 43 wherein the activator is imidazolium triflate.

85. The method of claim 1 wherein B is devoid of exocyclic amine protection.

25 86. The method of claim 12 wherein B is devoid of exocyclic amine protection.

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87. The method of claim 18 wherein B is devoid of exocyclic amine protection.

88. The method of claim 24 wherein B is devoid of exocyclic amine protection.

5 89. The method of claim 29 wherein B is devoid of exocyclic amine protection.

90. The method of claim 43 wherein B is devoid of exocyclic amine protection.

10 91. The method of claim 84 wherein B is devoid of exocyclic amine protection.

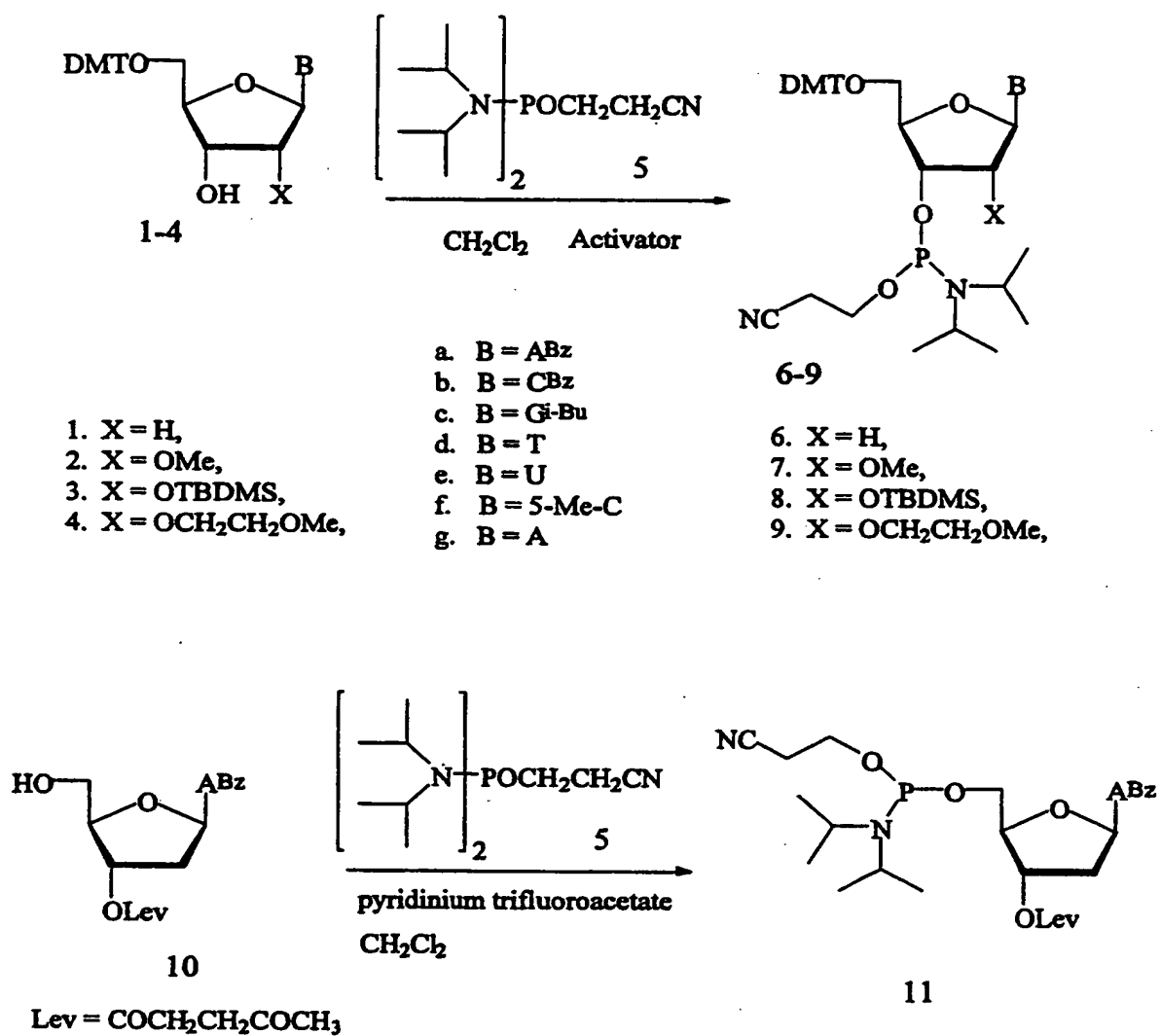
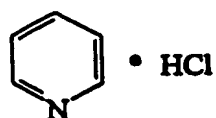


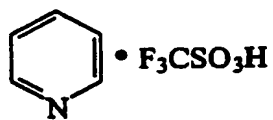
Figure 1



A, pyridine hydrochloride



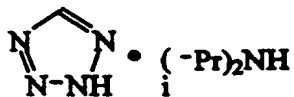
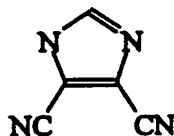
B, pyridinium trifluoroacetate



C, pyridinium triflate



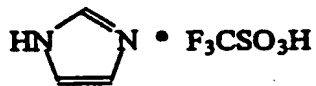
D, tetrazole

E, diisopropylammonium
tetrazolide

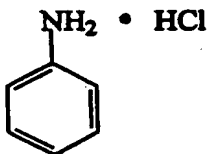
F, 4,5-dicyanoimidazole



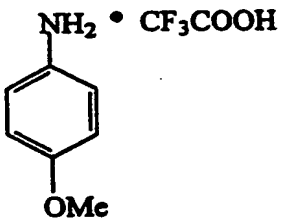
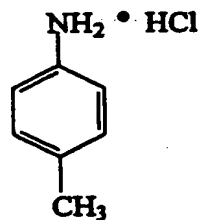
G, imidazole hydrochloride



H, imidazolium triflate

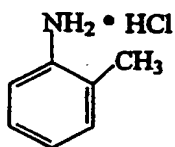


I, aniline hydrochloride

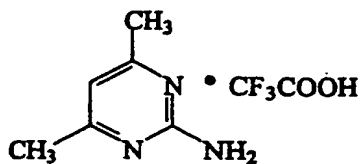
J, p-anisidinium
trifluoroacetate

K, p-toluidine hydrochloride

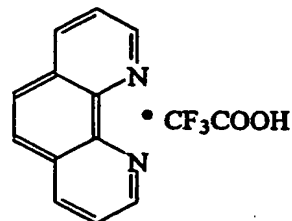
Figure 2



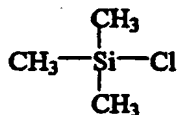
L, o-toluidine hydrochloride



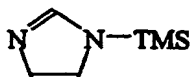
M, 2-amino-4,6-dimethylpyrimidine trifluoroacetate



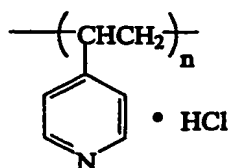
N, 1,10-phenanthroline trifluoroacetate



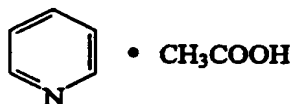
O, chlorotrimethylsilane



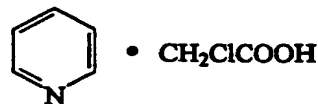
P, 1-(trimethylsilyl)imidazole



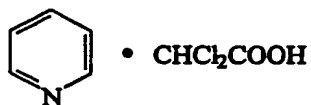
Q, poly(4-vinylpyridine hydrochloride)



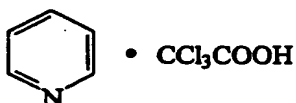
R, pyridinium acetate



S, pyridinium chloroacetate



T, pyridinium dichloroacetate



U, pyridinium trichloroacetate

Figure 3

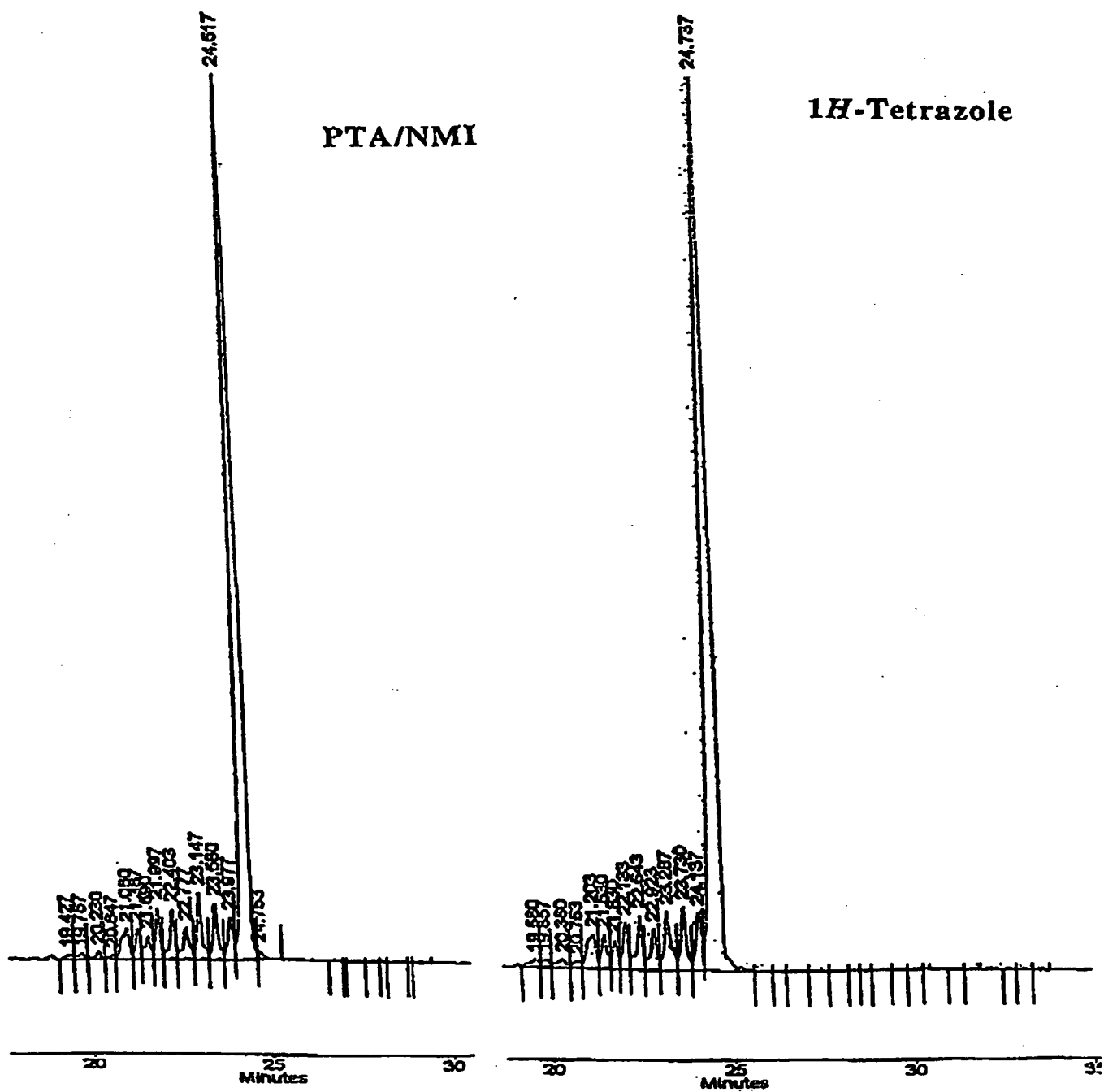


Figure 4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/12251

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C07H 21/00

US CL : 536/22.1, 23.1, 25.3, 25.33, 25.34, 25.4, 25.6

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/22.1, 23.1, 25.3, 25.33, 25.34, 25.4, 25.6

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS online

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4,760,137 A (ROBINS et al) 26 July 1988, cols. 4, 5 and 6.	1-91
Y	US 4,997,926 A (HAERTLE et al) 05 March 1991, cols. 16-20.	1-91
Y	US 5,208,327 A (CHEN) 04 May 1993, see cols 3-7.	1-91

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

01 OCTOBER 1999

Date of mailing of the international search report

07 OCT 1999

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